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Annual Research Report

A Research and Training Program in Tropical Veterinary Medicine  
(AID-csd-1947, in Colombia)

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## NARRATIVE SUMMARY OF ACCOMPLISHMENTS AND UTILIZATION

A group of blood diseases, primarily of cattle, transmitted by ticks and other biting insects, were selected for study because of their world-wide incidence and importance. The causative agents involved in these diseases, closely resemble similar infections of horses, sheep, and swine, as well as man, so that studies and accomplishments may actually have broader application than to just cattle.

Our research has been directed toward increasing the efficiency of cattle production through improved methods for the control of babesiosis, anaplasmosis and trypanosomiasis. Opportunities for the development of new methods for disease control become apparent through the better understanding of each disease. The techniques of epidemiology, microbiology, pathology and immunology facilitate the understanding and provide the tools for improved control.

A survey of these diseases and their incidence in Colombia, clearly demonstrated the presence of problem areas. The high incidence of these blood diseases throughout Colombia below the 5,000 foot elevation establishes and identifies the problem. The trends and incidence of infection are believed consistent throughout the tropics where elevation, and rainfall are similar. Studies of pathologic changes and the pathogenesis of infection has further established the importance of these diseases, and indicated what may be expected when infection occurs. Vectors including flies and ticks have been studied with respect to their role in the epidemiology of these blood diseases.

Progress in developing methods for improved control has been significant. Diagnostic serologic tests have been developed, and adapted for use in Colombia enabling us to identify infection in non-apparent carriers, thus better understanding incidence, and the interaction of the causative agents.

Vaccines have been tested, and some have resulted in the establishment of immunization regimes which offer the hope of significantly reducing live-stock losses from anaplasmosis and babesiosis. A study of the use of an integrated control program for blood, internal, and external parasites of cattle on the North Coast of Colombia (ICA - Turipana - Alt. 70 ft.) has demonstrated reduced production losses and increased net monetary returns.

New and effective therapeutic compounds have been and are being tested against both anaplasmosis and babesiosis. One drug (4A65) has been shown to be effective against both anaplasmosis and babesiosis. Two or 3 injections with this drug may replace the previous 12 injection series, or a 60 day feeding period. These results are so recent, however that confirming data will be required before widespread use can be recommended.

The shortage of U.S. veterinarians with training and experience in this area dictated a second phase of our program, that of graduate training

to develop the capability and interest in careers devoted to solving the disease problems of the tropics. A total of 10 U.S. graduate students have enrolled in this program since its beginning, 7 in microbiology, 2 in pathology, and 1 in parasitology. Two students have completed the Masters program, and one the Ph.D. program. Two are currently working on the Ph.D. degree, and 5 on the Masters degree. The approach whereby these students complete their course work in the U.S. and do their research in Colombia has worked well. An added phase of our training program in Colombia has been the supervision of students from South American countries both in Colombia by staff stationed there, and in the U.S. by Texas A&M staff. The broad range of participation on the part of student and staff, both from the United States and South America has established a rapport which has strengthened the veterinary programs in both Colombia and at Texas A&M.

## GENERAL BACKGROUND

Disease control is a basic pre-requisite for livestock survival and production in any locale, but becomes a major consideration and a decisive factor in the tropics, where environmental factors favor the development and transmission of animal disease. Historically the validity of this statement has been established in nearly every instance where high producing livestock have been introduced into the tropics.

Many tropical and sub-tropical areas of the world are noted for profuse forage and roughage production, unsuited for human food but capable of utilization by ruminant ungulates which can convert this resource into a high quality protein for human consumption. This production could contribute to the health and economic well being of millions of people now living on marginal diets, under poverty conditions. A major limiting factor, preventing livestock production, in these areas is animal disease. A major group of these diseases comprise the blood parasitic diseases of cattle including anaplasmosis, theileriasis (East Coast Fever), babesiosis (piroplasmosis), and trypanosomiasis. Wilson, et al. (Bulletin World Health Organization, 28, 595-613) estimates that the area in Africa virtually devoid of cattle, as the result of trypanosomiasis alone, exceeds the size of the United States. He estimates that this area could support 125,000,000 head of cattle were it not for this devastating cattle disease. Control of this disease plus the other blood parasitic diseases mentioned above would produce an economic - social impact on the areas so affected, that could hardly be measured. Such a step could rival, if not surpass, the so-called "Green Revolution" that has so significantly contributed to cereal grain production in recent years.

Past research efforts have contributed greatly to our knowledge of these diseases, but more information is needed to provide workable, practical control programs.

It was with this background, that our original project was submitted to AID for funding. The research proposal was aimed primarily at the applied, production oriented aspects, but in so doing it was recognized that basic studies would be required to provide the groundwork for applied techniques. General objectives of our program were the following:

1. To develop diagnostic techniques for the purposes of identifying the problem, and studying the epidemiologic aspects for possible control procedures.
2. To develop immunization systems to curb losses, and reduce the severity of infection.
3. To investigate therapeutic agents for their use in:
  - a. Treating sick animals and preventing death.
  - b. Eliminating the infection on a herd basis from both the vertebrate and invertebrate hosts.
4. To develop an integrated control program based on scientific facts uncovered by the above studies.
5. To enroll veterinarians in graduate programs in Tropical Veterinary Medicine, for research on and application of disease control techniques.

#### PROJECT OBJECTIVES

(As Submitted January 1968 With Minor Modifications)

1. To conduct research on tropical diseases aimed at developing information and methods for the control of these diseases in order to produce more beef in developing countries, with

initial emphasis on arthropod born blood diseases (anaplasmosis, babesiosis, theileriasis, and trypanosomiasis).

A. Anaplasmosis (Specific Objectives).

- a. To evaluate, under field conditions, vaccines presently available.
- b. To develop a more effective killed vaccine.
- c. To evaluate premunization methods as a means of prophylaxis.
- d. To establish the cattle passages required for reversion of the attenuated Anaplasma to virulence.
- e. To measure possible antigenic variations among Anaplasma organisms.
- f. To investigate the prevalence of disease and natural transmission by arthropod vectors.
- g. To investigate the use of systemic insecticides and other vector control measures as preventive measures.
- h. To evaluate therapeutic compounds for treatment.
- i. To conduct pathogenesis studies.

B. Babesiosis (Specific Objectives).

- a. To develop an effective immunological approach.
- b. To determine the prevalence of disease and the potential number of vectors in various geographic areas.
- c. To evaluate a tick eradication program as a means of Babesia control.
- d. To investigate various ixodocides in achieving tick control.
- e. To measure the productivity of infected cattle as compared to non-infected animals.
- f. To evaluate therapeutic compounds and assess their relative value in treating acute and chronic infections, and in eliminating the carrier state.
- g. To develop serologic tests for the detection of latent as well as acute infection.
- h. To determine the antigenic variations of Babesia organisms, by serologic and other means.
- i. To determine the Babesia incidence in cattle and wild fauna, and the role of wild life in maintaining infection.
- j. To develop non-bovine sources of Babesia organisms from ticks and tissue culture for use in vaccines.
- k. To conduct pathogenesis studies.

C. Other related blood diseases, including Trypanosoma and Theileria infections have been studied.

2. To train graduate students in research methods applicable to tropical diseases and to collect information, specimens, and illustrations for use in this training program.
3. To provide trained faculty and staff to operate veterinary programs

in developing countries, and to serve as consultants to related tropical disease problems.

#### CONTINUED RELEVANCE OF OBJECTIVES

Many of the objectives listed regarding anaplasmosis and babesiosis have been accomplished, with techniques and information evolving which offer hope for significant improvement in present control practices. Even so, many important links in our complete understanding of these 2 diseases are missing and will require continued research.

As the research on babesiosis and anaplasmosis proceeds to the demonstration of methods which provide effective control more attention can be given to trypanosomiasis, theileriasis or other parasitic diseases for which control techniques are relatively inadequate.

#### ACCOMPLISHMENTS TO DATE

During the past year, studies have been made which contribute directly to the objectives as listed above and in addition some research has been conducted on trypanosomiasis.

##### Anaplasmosis:

- a. An integrated control program for blood, internal, and external parasites of cattle on the North Coast (ICA - Turipana - Alt. 70 ft.) of Colombia has increased production and a net monetary return. The study involved the use of 78 indigenous 4 week old calves, randomly selected and distributed into one control and three treatment groups. Calves (Group I) were injected 1 time with blood containing A. marginale, B. bigemina and B. argentina followed by specific drug therapy to reduce the severity of infection and encourage immunity against hemoparasitic diseases. Calves (Group II) were treated twice with specific drug therapy to prevent

hemoparasitic diseases, while calves in Groups III and C were not treated against hemoparasites. Calves (Groups I, II, and III) were given oral drugs against internal parasites and sprayed topically against external parasites at 34 and 17 day intervals respectively, while calves (Group C) were not treated. Twenty-two parameters were measured in the experiment which had a duration of 257 days. The results of the total weight gains, net returns and mortality rates (%) are given in the table below.

	I	II	III	C	Significance
No of Calves	19	20	20	19	
Total Weight Gain (lbs)	6118	5854	4880	3236	P<0.01
Net Return (U.S. \$)*	1480	1418	1149	744	P<0.01
Mortality Rates	0	5	10	26.32	

\* Colombian market prices at P. \$9.80/kg or U.S. \$0.23/lb

From these data, it is apparent that control programs for Groups I and II are far superior to those of Groups III and C. When blood, internal and external parasites were controlled, highly significant net returns and weight gains resulted. On the basis of this data it may be concluded that such control is economical and productive, yielding highly significant net monetary returns.

- b. A serologic method has been developed to measure Anaplasma antigens in small amounts. Using this technique it is now anticipated that a more efficient approach will be possible to purify and detect antigens for use in a killed vaccine.
- c. A detailed study has been completed, comparing premunization methods involving virulent Anaplasma marginale, an attenuated A. marginale, and A. centrale, in adult cattle, in intact calves, and splenectomized calves. The attenuated A. marginale and A. centrale were equally mild in adult cattle and in intact calves, however reactions in splenectomized calves

were less severe with the attenuated A. marginale organism. Previous studies have shown that complete protection follows the use of the attenuated A. marginale, whereas protection is only partial or relative following the use of A. centrale. There are A. marginale variants (such as occur at Turipana, north coast of Colombia) which apparently will overwhelm the attenuated A. marginale. In these areas the only satisfactory protection in the past has been produced by virulent A. marginale.

- d. An interesting and possibly highly significant observation has been made relative to reversion of the attenuated A. marginale. Previous studies have shown complete reversion after 12 serial passages in splenectomized calves. Checks of remaining infectivity among adult cattle vaccinated with the attenuated A. marginale 11 months previously, not only showed the infection present but indicated the presence of an Anaplasma equally as virulent as a field isolate of A. marginale. The implication of this finding is that during the 11 months the attenuated organism reverted to virulence without serial passage. If later experiments confirm this we might well expect that the attenuated A. marginale would be capable of establishing infections equal in virulence and hence, protective capabilities, to the normal virulent field strain, but without the severe acute infections which occur in the field strain.
- f. The horseflies Tabanus nigrovittatus, and T. sulcifrons, have been used in transmission studies. Anaplasma was isolated in the horsefly up to 24 hours after feeding on an Anaplasma infected calf.
- g. See Anaplasmosis section a.
- h. A major effort has been made during the past year to more thoroughly evaluate 2 new drugs which show promise as therapeutic agents for anaplasmosis, along with the more conventional tetracycline therapy. The

following compounds have been used:

1. Gloxazone (Alpha Ethoxyethylglyoxal dithiosemicarbazone) (356 C 61).
2. Imidocarb (3,3'-Bis-(2-imidazolin-2-yl)-carbanilide dihydrochloride (4A65).
3. Chlortetracycline and oxytetracycline.

The tetracyclines administered orally for 60 days or injected daily for 10-14 days have been reported to eliminate Anaplasma infection in intact cattle. In splenectomized calves our results were sporadic. The tetracyclines while of proven value are time consuming, expensive, and not 100% effective in eliminating infection. Gloxazone (356 C 61) given alone, while active is inadequate to eliminate infection in subtoxic amounts. Combined therapy, using 356 C 61 and oxytetracycline is highly effective, and has consistently removed carrier infections in splenectomized calves following only 3 I.V. treatments at 24 or 48 hour intervals at the level of 11 mg/kg oxytetracycline and 5 mg/kg 356 C 61. Imidocarb (4A65) given alone or in combination with 356 C 61 has proven effective in eliminating Anaplasma infection, but not without some toxic side effects. An intramuscular (i/m) dose of 2 mg/kg 4A65 and 5 mg/kg 356 C 61 or 5 mg/kg 4A65 and 2 mg/kg 356 C 61 given on 3 successive days has consistently eliminated Anaplasma infections in splenectomized calves. An i/m or s/c dose of 4, 5 or 6 mg/kg 4A65 given 3 times at 24 or 48 hour intervals has also eliminated the infection. Results with 4A65 alone are however, more sporadic and success is less predictable. Several old cows have died of acute nephrosis 28 to 34 days after treatment with 4A65. We cannot at this time be certain that death was drug related but there is evidence to suggest this. Further studies are in progress.

Prevention by treatment has been explored using low levels of chlortetracycline (0.1 mb/lb, and 0.5 mb/lb) added to the ration and

administered orally. The lower level (0.1 mg/lb) was unsatisfactory, and even though the higher level (0.5 mg/lb) was effective it is doubtful if it would be economically feasible. A monthly and weekly s/c injection of 4A65 has been used in the presence of continuous exposure to evaluate the possible use of this method of prevention. The monthly injections of 5 mg/kg 4A65 in 1 instance prevented infection, and in another instance markedly reduced the severity of infection; this approach might prove useful, however toxic problems must be evaluated.

- i. In tropical areas of Colombia, bovine anaplasmosis and babesiosis occur with a high frequency of concurrent infections. These concurrent infections exacerbates the severity of clinical illness as compared to singular infection, and frequently lead to death, even in indigenous cattle. The mechanism of dual infections is not known. Studies have been made of the clinical disease, serologic response and pathologic manifestations of cattle concurrently infected with Anaplasma marginale and Babesia bigemina. The results indicate that a concurrent infection of anaplasmosis and babesiosis have an additive effect in producing higher parasitemias, more severe anemias and a higher mortality.

Babesiosis:

- a. Babesia bigemina was subjected to varying doses of gama radiation to determine the influence of this treatment on the organisms ability to induce infection and or immunity. Some calves receiving B. bigemina irradiated at levels of 36-42 kRad did not develop progressive infections. Progressive Babesia infections were prevented by exposure of the organism to 48 kRad or higher. A degree of acquired resistance to infection with non-irradiated B. bigemina developed in calves after 1 inoculation with B. bigemina irradiated at 48-60 kRad. A standard dose of diluted or irradiated organisms

could be of use as a vaccine to produce a low degree of infection of cattle with a high degree of immunity without drug therapy.

- b. Vector studies have been and are continuing to be made in cooperation with the U.S.D.A. - Division of Entomology Research, Kerrville, Texas, at their sub-station in Nuevo Laredo, Mexico. An intact calf infested with Boophilus microplus has been treated with 10 mg/kg, 4A65, at a time when adult ticks were starting to feed. Preliminary evaluation of results shows the drug to have no influence on the ticks feeding, on the egg mass from engorged females, or on the viability of the resulting larvae.

A study was made of chronic wasting disease of cattle (Secadera) in the Eastern plains of Colombia. Calves up to 6 months of age were not affected. Cattle 7-18 months old, having this syndrome, showed a high degree of correlation between their physical appearance and the presence of blood and internal parasites, nutritional deficiencies, and poor management. In cattle 19 months old and older, these same relationships were noted but to a greater extent. Based on these studies Secadera is thought to be a complex of several disease causing agents and poor management, of which blood parasites are one of the foremost factors. Hemo-parasite control would have a large impact on increasing beef production in this tropical zone.

- f. Treatment studies for babesiosis have been conducted using a Babesia rodhaini model in mice. A total of 19 compounds or combinations of drugs have been tested in mice for possible activity against Babesia. Three compounds have shown activity, others were ineffective. Imidocarb in addition to being effective against Babesia is also proving effective against Anaplasma. The possibility exists that the rodhaini mouse model might detect drugs having anaplasmaicidal activities. Drugs that

are effective against rodhaini will later be tested in calves as treatments for anaplasmosis.

- g. Babesia bigemina and Babesia argentina antigens were isolated from infected red blood cells and are routinely used in a complement fixation serologic test in our laboratory. The need is urgent for a rapid field serologic test for the detection of babesiosis of cattle. Work is in progress on a rapid card test (RCT) similar to that being used by the USDA for anaplasmosis. Some preliminary results with the RCT were encouraging and methods to eliminate the inherent problems of specificity are being studied.
- h. Cattle transported from one area to another will often suffer attacks of blood diseases which are frequently fatal. A study was initiated to measure the differences between the tick induced and laboratory induced infections. The tick infections were isolated from the blood of cattle infested with infective ticks. The laboratory type of Babesia bigemina organisms was isolated from the blood of cattle infected by the inoculation of blood. The laboratory type of organisms induced more relapses, higher parasitemias and greater serologic response. The tick induced infections retarded weight gains, produced more severe anemias, and resulted in higher body temperatures. These preliminary studies suggest that variations occur within the same species of organism. If so, production of vaccines, should attempt to incorporate all types of the same species of blood parasites.

Studies have been made of similar and dis-similar antigenic characteristics of Babesia bigemina and Babesia argentina to determine if one organism will protect against the other and vice versa. On the basis of these experiments, two types of antigenic characteristics were

found: (1) species specific or distinct antigenic characteristics for each species of organism (2) common genus or similar antigenic characteristics for both organisms. In addition, two groups of calves were infected with Babesia bigemina and Babesia argentina respectively, and 176 days after infection, they were challenged with the same and the heterologous parasites. It was found that Babesia bigemina and Babesia argentina parasites gave partial cross-protective immunity against one another. Babesia argentina gave more protective immunity against B. bigemina than the contrary, therefore singular vaccines for protection against babesiosis should include B. argentina rather than B. bigemina.

- i. All efforts to infect deer with Babesia by tick or needle challenge have been unsuccessful.
- j. Because B. bigemina only lives and grows in either cattle or Boophilus ticks, research efforts are limited due to the use of expensive cattle as research animals. Tissue culture systems derived from the lymph nodes, hemal lymph nodes and spleens of experimentally infected and non-infected calves have been established by various techniques. The non-infected cattle tissue culture systems were inoculated with cattle blood infected with B. bigemina. Rudimentary evidence suggests that the organism survives and possibly may even proliferate in tissue culture systems, inoculated with blood infected with B. bigemina although the experiment is still in its primary stages. Various attempts have been made to establish tissue culture systems derived from infected Boophilus microplus larval ticks, but none have been successful.

In order to circumvent the problems associated with the use of cattle blood origin vaccines for blood diseases, a study is in progress to establish a source of viable Babesia argentina organisms from infected

Boophilus microplus ticks. All attempts to transmit babesiosis by tick origin material have failed, probably due to the small numbers of infective particles present in the inoculum. Further studies are in progress to produce higher Babesia infections of the ticks.

k. See Anaplasmosis, section i.

Trypanosomiasis:

A total of 64 serial passages of Trypanosoma theileri have been made on blood agar slants. T. theileri has been successfully cultivated in tissue culture media, and in embryonated eggs. An isolation of T. theileri has been made in a splenectomized calf from a cow showing trypanosomes in blood smears. Studies of the T. theileri incidence, have revealed this organism to be widespread in Texas. Cultures made on blood agar slants from 235 cattle in 6 vegetational areas of Texas have showed 63.4% to be positive for T. theileri. Infection rates were much higher in animals over a year of age.

The prevalence, incidence and economic importance of trypanosomiasis (T. vivax) to the Colombian cattle industry are unknown primarily due to inadequate detection techniques. Studies are in progress to develop a specific serologic test and indirect fluorescent antibody technique, for the detection of T. vivax, infections. This test is particularly practical because small blood samples may be taken by pricking the skin and absorbed onto filter paper for processing. In this manner, the samples may be sent to the laboratory in a letter. Preliminary results from this serologic test indicate that it will result in a useful detection system, which will be employed to delineate the importance of cattle trypanosomiasis in Colombia.

SUPPORTING EVIDENCE

Attached to this report is Appendix IV and V which provides a detailed description of the experimental work.

## RESEARCH DESIGN

The emphasis in Colombia is on babesiosis and trypanosomiasis with minor considerations given anaplasmosis. In Texas the major emphasis is on anaplasmosis and babesiosis with minor considerations given trypanosomiasis.

There have been 2 full time staff members living in Colombia, plus 3 to 5 Research Assistants (working for advance degrees), and 1 full time staff member residing in Texas, engaged in research.

The work in both Colombia and Texas compliments the other, and contributes a greater understanding and appreciation of the problem, by providing an up to date exchange of information between all professional staff which facilitates implementation of research findings and allows rapid confirmation, or second party examination of research findings. Our present approach has worked well, however there is room for improvement. An opportunity for the research staff to assemble more frequently than the present annual review would facilitate the exchange of information. Greater participation by research staff in field trials both in Colombia and the U.S. might increase the yield of information and accelerate progress.

The research approach is basically sound, and workable to the type work engaged in. We are basically following the procedures and plans as outlined in our previous proposals.

## DISSEMINATION AND UTILIZATION OF RESEARCH RESULTS

A list of titles and abstracts of presentations made to scientific gatherings and later published, and other publications are listed in Appendix I. The relationship of disease to productivity as applied primarily to this project is presented in Appendix II.

The research capabilities established first at ICA, and later CIAT have been used not only by our staff, but also by Colombian veterinarians and

students working on similar problems and often in cooperation with our team. This project has markedly stimulated local interest in these problems and has contributed to a better appreciation for what can and should be done. The Colombian government (ICA) has constructed a modern laboratory at Turipana for the study of tropical hemotropic diseases.

Research reports, concerning the efficacy of 4A65 has stimulated company officials (Burroughs Wellcome) to seek clearance from FDA for further experimental field testing in the U.S. Field trials based on premunition, and treatment are underway in Colombia, utilizing past research experience.

The program has been fortunate in working with Colombian veterinarians with common interests. There have been a large number of these people who have worked with Texas A&M staff, as students, and associates both in Colombia and Texas who continue to cooperate and assist in our program. These men have been acknowledged and their work recognized in the 1971 and 1972 reports, for both Colombia and Texas.

#### STATEMENT OF EXPENDITURES AND OBLIGATIONS AND CONTRACTOR RESOURCES

Funds have been spent both in the U.S. and in Colombia for budgeted items including manpower, equipment, travel, etc. In Colombia local expenditures for local wages, travel, etc., are made by CIAT on our behalf, and CIAT is periodically reimbursed from the AID account maintained at Texas A&M by the Office of International Programs. All other purchases, and staff salaries (in Texas and Colombia) are handled through this office. Most items of equipment and supplies for use in Colombia are first delivered to Texas A&M for transshipment to Colombia. A possible improvement would be the purchase of all equipment and supplies for use in Colombia, through CIAT, utilizing the CIAT purchasing and shipping agent in the U.S., with periodic

reimbursement to CIAT. Such a plan might merit some thought and consideration for the future.

During the past year the entire staff, previously living in Bogota were moved to Cali, as a part of the transfer of our project from ICA (Instituto Colombiano Agropecuario) to CIAT (Centro Internacional de Agricultura Tropical). This has been a year of transition, with the need for establishing a new laboratory, with new technical personnel, and labor force.

In the coming year it is anticipated that Dr. R. A. Todorovic will return to the U.S. in July 1972, after almost 4 years in Colombia. Dr. Adams will return in January 1973, after almost 5 years in Colombia. It is presently anticipated that Dr. T. J. Galvin will be going to Colombia in August 1972, and that Dr. D. E. Corrier will be going to Colombia in October 1972 or soon thereafter. After their return to Texas, Drs. Adams and Todorovic will remain on the project half time, with the other half of their time being taken by the departments of Pathology and Microbiology for teaching. Drs. Maurer and Kuttler will remain in College Station.

A budget statement for 1971-72 outlining expenditures and obligations for each of the major inputs and the major targets is given, see Appendix III.

#### WORK PLAN AND BUDGET FORCAST FOR THE COMING YEAR

In general the programs at Texas A&M and Colombia will be oriented toward the completion of present projects and utilization of past research results to evolve systems and techniques for the more efficient and economical control of hemotropic diseases of food producing ungulates.

Work in Colombia will include studies on all 3 major hemotropic diseases; anaplasmosis, babesiosis, and trypanosomiasis, but with an emphasis on the last two. Personnel involved in these studies include 4 Research Assistants

(Drs. Wyss, Platt, Craig and Thompson) and 4 staff members (Drs. Adams, Todorovic, Galvin and Corrier).

Research on hemotropic diseases at Texas A&M in the past has been productive, even though handicapped to some extent in that field studies on all agents are impossible. Even though the pathogenic cattle Babesias and T. vivax are not present in Texas, studies with Babesia, trypanosomes, and Anaplasma are in progress. In addition, studies are being conducted on a Theileria, not known to be present in South America, but which is morphologically similar to the causative agent of East Coast Fever, occurring in East Africa. Research emphasis is being placed on anaplasmosis, largely because it is a local problem, field studies are possible and the agent is readily available.

It is anticipated that data will be completed on the field trials at Turipana in which tests are being made of immunization and therapeutic programs for control of the hemotropic diseases. A diagnostic test for trypanosomiasis may be available for use in field surveys, on large numbers of samples. The study of tissue culture growth of Babesia will be completed, and the study of growth or the presence of Babesia organisms in tick tissues will be completed.

Additional pathologic studies will be made of anaplasmosis, babesiosis and trypanosomiasis. Work is continuing on tissue culture growth of Anaplasma, and while as yet unsuccessful it is hoped that future work may be more encouraging. Work will proceed using the new therapeutic agents for treatment of anaplasmosis and babesiosis. It is anticipated that by the end of the year some firm recommendations for control will be possible. Increased emphasis will be placed on the production of a more purified killed Anaplasma vaccine. Future field trials will be conducted with the attenuated Anaplasma vaccine.

The movement of personnel from Colombia to Texas and their replacement during the coming year will be a disruptive influence, but work continuity will be encouraged by staggering these moves so that we will not have all new people at any one time, allowing those arriving in Colombia time to become adjusted before the next new staff member arrives. Facilities at Texas A&M may become cramped, with the added staff, but accommodations for this addition will be made.

A budget statement for 1972-73 showing planned expenditures for each of the major inputs and the major work targets is given, see Appendix III.

**APPENDIX I**

## APPENDIX I

KUTTLER, K. L., ZARAZA, H. AND ROBERTS, E. D.: Hematologic and Clinical Response to Anaplasmosis Vaccines and the Comparative Efficacy of These Vaccines, As Measured by Field and Experimental Challenge. Proceedings of the 5th National Anaplasmosis Conference. February 28-29, 1968, Stillwater, Oklahoma.

Twenty, 3-month-old calves were divided in 4 equal groups. Group 1 was inoculated with an attenuated Anaplasma marginale, group 2 received an A. marginale adjuvant vaccine, group 3 was infected with virulent A. marginale followed by treatment, and group 4 remained as unvaccinated controls. All animals were moved into an Anaplasma endemic zone 3 months later and allowed to undergo natural field challenge. Evidence of acute anaplasmosis was observed in all calves, except those premunized by virulent A. marginale. No significant evidence of protection was produced by either the attenuated A. marginale or the adjuvant vaccine when compared to the unvaccinated controls. The group premunized with virulent A. marginale failed to respond to natural exposure.

Hematologic response to virulent, attenuated and killed A. marginale vaccines was measured in 18 mature cattle divided into 3 groups. The group receiving virulent A. marginale was treated 25 days after infection (Burroughs Wellcome Compound 356-C-61). No death losses occurred in this group, but moderate infections were observed to result in a significant reduction of PCV. The attenuated A. marginale vaccine produced a low level parasitemia, a marked serological response, as measured by the complement-fixation test (CF), and a very slight drop in PCV, which was not significantly different from values observed in an unvaccinated, non-infected, control group. The group receiving adjuvant vaccine showed only a low level, transient, CF serological response.

An experimental challenge was administered 8 weeks after vaccination to cattle receiving the attenuated and adjuvant vaccines along with a group of 5 unvaccinated controls. All controls reacted to challenge with severe acute signs of anaplasmosis. One animal was allowed to die, a second would probably have died, had it not been treated. Cattle receiving the attenuated vaccine showed no signs of active infection resulting from challenge. Cattle receiving the adjuvant vaccine reacted to challenge, but less severely than did the controls.

ZARAZA, H., KUTTLER, K. L. AND ROBERTS, E. D.: Efectos de la Descarga Natural de Anaplasma marginale en Terneros Vacunados y no Vacunados. Instituto Colombiano-Agropecuario, Revista ICA Vol., IV., No. 3 (September 1969). (Spanish Translation of Above).

ZARAZA, H., KUTTLER, K. L.: Respuesta Hematologica y Clinica a Diferentes Vacunas de Anaplasmosis y la Eficacia Comparativa de estas, Evaluadas por la Inoculacion Experimental. Revista ICA, Vol. III, No. 4, (Diciembre De 1968) 323-331. (Spanish Translation of Above).

KUTTLER, K. L., ADAMS, L. G., AND ZARAZA, H.: An Epidemiologic and Geographic Survey of Anaplasma marginale and Trypanosoma theileri in Colombia. 106th Annual AVAMA Convention, July 1969, Minneapolis, Minn., Journal of the Veterinary Medical Association, 154, (June 1, 1969), 1398, (Abstr.).

Anaplasmosis complement-fixation tests, packed cell volumes, and stained blood smears were made on 603 cattle located at 5 experiment station farms in Colombia. These farms were situated in differing climatic zones varying from 2,600 meters to 13 meters in altitude and from 13°C to 28°C in mean temperature. Specific reference was made to breed susceptibility, the influence of age, and climatic condition on the incidence and severity of infection.

A direct correlation was noted between mean temperature and incidence of anaplasmosis. At 13°C the incidence was nil, whereas at 28°C over 90% infection was noted. The mean temperature is directly associated with altitude.

Incidence of infection in enzootic areas was generally greater in older animals, but the effect of infection as characterized by anemia was more noticeable in young animals. The incidence of anaplasmosis in European breeds did not appear greatly different when compared to native and Zebu cattle, but in some instances PCVs were significantly lower in European breeds. This was most marked at the lower elevations.

Blood cultures for T. theileri from 71 cattle at 2 experiment stations resulted in a pattern of infection similar to anaplasmosis. A high incidence of infection was noted at the lower elevation with a high mean temperature and no evidence of infection at 2,600 meters with a low mean temperature.

KUTTLER, K. L., ADAMS, L. G. AND ZARAZA, H.: Estudio Epizootiologico del Anaplasma marginale y et Trypanosoma theileri en Colombia. Revista ICA, Vol. V., No. 2 (June, 1970) (Spanish Translation of Above).

KUTTLER, K. L. AND ZARAZA, H.: A Preliminary Evaluation of a Dithiosemicarbazone for the Treatment of Anaplasmosis. Res. in Vet. Sci., 2, (July 4, 1970), 334-338.

Trials were conducted on 3 splenectomized calves treated with a single intravenous (i.v.) inoculation of a dithiosemicarbazone (356 C 61) using 5 mg./kg., at different stages of induced anaplasmosis infection. When compared to an untreated control this compound was effective in reducing the severity of the infection. Haematological response was least severe in the animal receiving treatment before signs of parasitemia or a decrease in packed cell volume had occurred.

Treatment with compound 356 C 61 (5 mg./kg. i.v.) of 5 splenectomized calves, and 6 intact adult cattle, early in the course of an artificially induced Anaplasma marginale infection prevented death loss and reduced the severity of the subsequent reaction when compared with non-treated controls.

KUTTLER, K. L. AND ADAMS, L. G.: Comparative Efficacy of Oxytetracycline and a Dithiosemicarbazone in Eliminating Anaplasma marginale Infection in Splenectomized Calves. Res. in Vet. Sci., 2, (July 4, 1970), 339-342.

Comparisons between oxytetracycline and a dithiosemicarbazone (356 C 61) were made in 11 splenectomized, Anaplasma marginale infected calves. Oxytetracycline was administered at the rate of 11 mg./kg. intravenously (i.v.) for 5 and 10 consecutive days. Compound 356 C 61 was administered at the rate of 5 mg/kg i.v. for 5 and 10 consecutive days.

Compound 356 C 61 appeared to be relatively more effective in the treatment of anaplasmosis, as indicated by the relative increase in packed cell volume (PCV) following treatment, and by the apparent elimination of the carrier status in animals receiving the 10 daily treatments. Compound 356 C 61 administered daily for 10 consecutive days resulted in rumen atony, tympanites and death.

ADAMS, L. G. AND KUTTLER, K. L.: Toxicity of Alpha-Ethoxyethylglyoxal Dithiosemicarbazone in Cattle. Am. J. of Vet. Res., 31, 1493-1495, (August 1970).

Alpha-ethoxyethylglyoxal dithiosemicarbazone, administered 10 consecutive days at the dose rate of 5 mg./kg./day, caused axonal and myelin degeneration of the vagus nerve in 2 of 7 calves. Of the 7 experimental calves, 6 died of tympanites.

KUTTLER, K. L.: Serial Passage of an Attenuated Anaplasma marginale in Splenectomized Calves. Presented at the 1969 Annual Meeting of the USAHA, Milwaukee, Wis., Proc. 73rd Ann. Meeting of the USAHA, Oct. 12-17, 1969, Milwaukee, Wis., 131-135.

Twelve serial passages of an attenuated Anaplasma marginale were made in splenectomized calves by blood inoculation. The severity of infection produced at the twelfth passage level in 4 splenectomized calves was compared to the infection occurring in 4 similar calves at a second passage level. Significantly higher parasitemias and lower packed cell volumes occurred in the twelfth passage group, suggesting an increased virulence. No deaths occurred among animals of the second passage group whereas 1 of 4 died in the twelfth passage group.

KUTTLER, K. L. AND ZARAZA, H.: Premunization With an Attenuated Anaplasma marginale. Presented at the 1969 Ann. Meeting of the USAHA, Milwaukee, Wis., 104-112.

An attenuated Anaplasma marginale infection has been established in 21 calves and 12 mature cattle. The resulting infections were found to be significantly less severe than virulent A. marginale in 12 calves and 5 mature cattle. A slightly milder response to the attenuated A. marginale occurred in calves at Bogota with a mean temperature of 14°C when compared to calves similarly infected at Palmira with a mean temperature of 24°C.

Calves and mature cattle previously premunized with the attenuated organism appeared to be immune to virulent challenge using a Texas isolate of A. marginale. Experimental and natural challenge with a Colombian isolate resulted in evidence of acute anaplasmosis in both vaccinated and non-vaccinated animals.

KUTTLER, K. L., GRAHAM, O. H., AND JOHNSON, S. R.: Apparent Failure of Boophilus annulatus to Transmit Anaplasmosis to White-Tailed Deer (Odocoileus virginianus), J. of Parasit., 57, (June 1971), 657.

Transovarial transmission of anaplasmosis occurred when two splenectomized calves were infested with unfed larvae of Boophilus annulatus but no evidence of infection was detected in 2 intact white-tailed deer after they were infested with other larvae of common origin. All attempts to isolate Anaplasma marginale from the 2 deer by transfer of blood into splenectomized calves were unsuccessful.

CORRY, G. L., KUTTLE, K. L.: Serological Activity of a Soluble Antigen of Theileria cervi. Submitted to "Experimental Parasitology".

Two basic cellular erythrocytic antigens were prepared from erythrocytes obtained from a white-tailed deer (Odocoileus virginianus), infected with Theileria cervi. The first antigen was prepared from erythrocytes lysed by freezing, the second from erythrocytes lysed with distilled water. The serologic activity as determined by the complement-fixation (CF) test was greater in the antigen lysed by freezing. Both antigens when solubilized at pH 7.2 using ultrasonic disintegration increased markedly in titer.

The two antigens were pooled and disrupted by ultrasonic disintegration in buffered mediums, ranging from pH 5 to pH 11. Optimal solubilization and serologic activity as measured with the CF test was obtained at pH 11.

The antigen solubilized at pH 11, was used to determine antibody in sera from infected deer by (CF) and by passive hemagglutination (PHA) tests. Both tests resulted in similar but not identical antibody titres.

A gel diffusion test and a ring (interfacial) test, gave no valid results.

INDEX DESCRIPTORS: Theileria cervi effect of soluble antigen on complement-fixation and passive hemagglutination.

Theileria cervi is a hemoparasite of the white-tailed deer (Odocoileus virginianus), first described by Schaeffler (1961). It was thought by Marburger and Thomas (1965) and Robinson et al (1967) to be a contributing factor of death losses among deer in Texas.

Other organisms of the same genus occur in different parts of the world, where they cause diseases of varying severity in domestic and wild animals.

Laboratory diagnosis of these diseases depends primarily, on microscopic detection of the parasites in stained smears. Serologic tests have been described by Schaeffler (1963), Kuttler and Robinson (1967), Kuttler et al (1967) and Gadir et al (1970). The antigens used in these tests were, for the most part, particulate. In this state, the cell membrane is most responsible for antigenic and serologic activity with the internal structure of the parasite cell not so greatly involved. It is not unreasonable to assume, that the cytoplasm of the parasite cells contains a mosaic of antigens, which when dispersed in molecular phase, i.e., when in solution are capable of more specific or sensitive reactions. Such antigens could find wide use in serologic tests, and possibly prove of greater value in elucidating the antigenic relationship of similar intraerythrocytic parasites.

The purpose of this work therefore, was to investigate the possibility of establishing a method of obtaining a soluble antigen from the erythrocytic stage of T. cervi, that would react with the homologous antibody in an *in vitro* system.

BISHOP, J. P. AND KUTTLER, K. L.: Infectivity and Immunogenicity of Irradiated Babesia rodhaini. Submitted to "The Journal of Protozoology".

Babesia rodhaini parasitized mouse blood exposed to varied doses of gamma radiation up to 30,000 r was inoculated into mice. Mice inoculated with non-irradiated B. rodhaini developed progressive infections and died 7 to 11 days after inoculation. Mice infected with B. rodhaini parasitized blood exposed to doses up to and including 22,000 r developed progressive parasitemias which were delayed in comparison to mice inoculated with nonirradiated B. rodhaini. Some mice receiving parasitized blood irradiated at 26,000 r did not develop progressive parasitemias. Progressive infections were prevented by exposure to irradiation at 30,000 r.

The results of two separate experiments revealed that one inoculation of parasitized blood exposed to 30,000 r or higher apparently stimulated a resistance to a challenge infection with nonirradiated parasitized blood. While 20 out of 20 control mice died as a result of challenging infections, 9 out of 28 mice previously exposed to irradiated parasitized blood survived.

The injection of irradiated nonparasitized blood did not produce a discernable acquired resistance to B. rodhaini. Presumably the irradiated parasitized blood was responsible for the development of acquired resistance to B. rodhaini.

ZARAZA, H., AND KUTTLER, K. L.: Comparative Efficacy of Different Immunization Systems Against Anaplasmosis. *Trop. Ani. Hlth. & Prod.*, 3, (1971), 77-82.

Animal response to anaplasmosis vaccination was measured using an attenuated organism, a killed adjuvant vaccine, and a virulent Anaplasma marginale. A total of 7 calves (2-4 months of age) and 5 heifers (18 months of age) received the attenuated organism; 8 calves were given the adjuvant vaccine; 7 calves were premunized with virulent A. marginale; and 7 calves remained as non-vaccinated controls. The animals were vaccinated at Tibaitata on the Bogota Savannah, and later moved to the north coast of Colombia, and anaplasmosis enzootic area.

All vaccination methods produced positive CF results. The live agents resulted in low parasitaemias in most instances, although the attenuated organism was particularly mild in the younger animals.

Protection from field challenge was observed in all calves premunized with virulent organism, and in two of five heifers premunized with the attenuated organism. All other vaccinated animals developed anaplasmosis which was equally as severe as seen in the non-vaccinated controls.

KUTTLER, K. L.: Efficacy of Oxytetracycline and a Dithiosemicarbazone in the Treatment of Anaplasmosis. Am. J. of Vet. Res., 32, (September 1971), 1349-1352.

The combination of a dithiosemicarbazone (356 C 61) and oxytetracycline proved more efficacious in the treatment of anaplasmosis than did either drug administered alone. The Anaplasma marginale carrier state in splenectomized calves was suppressed for as long as 120 days and was possibly eliminated by 3 injections of 356 C 61 (5 mg./kg.) and oxytetracycline (11 mg./kg.) given simultaneously at 48-hour intervals.

KUTTLER, K. L., GRAHAM, O. H., JOHNSON, S. R., AND TREVINO, J. L.: Unsuccessful Attempts to Establish Cattle Babesia Infections in White-tailed Deer (Odocoileus virginianus). J. of Wildlife Dis., 8, (Jan. 1972).

Attempts to induce a demonstrable cattle Babesia infection by feeding known infected ticks on two white-tailed (Odocoileus virginianus) deer were unsuccessful. The injection of known Babesia carrier blood into an intact and a splenectomized deer failed to result in evidence of infection.

All deer were checked for possible sub-patent infections by inoculating their blood into splenectomized calves at weekly intervals for 5 weeks following exposure, but no infections were produced in the calves.

Babesia infected ticks having undergone one generation on deer were unable to transmit infection to splenectomized calves on the succeeding generation.

KUTTLER, K. L.: Combined Treatment With A Dithiosemicarbazone and Oxytetracycline to Eliminate Anaplasma marginale Infections in Splenectomized Calves. Submitted to Research in Veterinary Science. Accepted for publication 1-3-72.

A total of 12 treatment schedules combining oxytetracycline and an alpha-dithiosemicarbazone (356 C 61) were tested on 36 splenectomized calves carrying Anaplasma marginale infections. Anaplasma infection was eliminated following the administration of 5 or 10 mg/kg 356 C 61 combined with 11 mg/kg oxytetracycline, and given 3 times at 24 or 48 hour intervals. Treatments employing lower levels, fewer injections, or at greater time intervals failed to eliminate infection.

Treated, splenectomized calves failing to show evidence of an A. marginale relapsing infection within 62 days were found to be free of infection on the basis of infectivity trials conducted an average of 87 days after treatment, and by re-inoculation with A. marginale an average of 164 days after treatment.

KUTTLER, K. L.: Promising Therapeutic Agents for the Elimination of Anaplasma marginale In the Carrier Animal. Presented at the 75th Ann. Mtg. - USAHA - Oklahoma City, Oklahoma, October 27, 1971 - To be published in Proceedings.

Two new drugs, a-dithiosemicarbazone (356 C 61) and 3,3'-Bis(2-imidazolin-2-yl) - carbanilide dihydrochloride (4A65) have been successfully used to treat splenectomized calves with anaplasmosis. Carrier infections were eliminated with 5 or 10 mg/kg 356 C 61 and 11 mg/kg oxytetracycline when given 3 times at either a 24 or 48 hour interval. In addition 5 mg/kg 356 C 61 plus 2 mg/kg 4A65 given 3 times at 24 hour intervals was effective in eliminating A. marginale infections. Levels of 4 and 6 mg/kg of 4A65 given 3 times at 24 hour intervals has proven successful in eliminating A. marginale infection.

KUTTLER, K. L.: Comparative Response of Premunization Using Attenuated Anaplasma marginale, Virulent A. marginale and A. centrale in Different Age Groups. Accepted for publication in Trop. Anim. Hlth. & Prod.

Premunizing infections using virulent Anaplasma marginale (VAM), attenuated A. marginale (AAM) and A. centrale (AC) have been induced in 4 mature cattle, 33 intact calves, and 38 splenectomized calves, for the purpose of comparing the relative response to these infections.

The VAM produced significantly more severe reactions in adult cattle, and splenectomized calves, and a slightly more severe response in intact calves; however, these animals were relatively more resistant to all three infections. There was no detectable difference between the reactions caused by AAM, and AC when measured in adult cattle and intact calves. Among splenectomized calves, however, the AAM infections resulted in a milder response as measured by the relative drop in packed cell volume and percent parasitemia. The CF response was significantly lower in the AC infection.

CARSON, C. A.: An Antigenic and Serologic Comparison of Two Virulent Strains and an Attenuated Strain of Anaplasma marginale. A Thesis submitted to the Graduate College of Texas A&M University in partial fulfillment of the requirement for the degree of Master of Science, August 1969.

An antigenic and serologic study was conducted using virulent strains of Anaplasma marginale from Texas and Colombia and an attenuated strain of Anaplasma marginale. Soluble antigens of the three A. marginale strains were compared by agar gel diffusion and immunoelectrophoresis. Serum proteins from calves infected with each of the three A. marginale strains were separated electrophoretically and reacted with rabbit anti-bovine serum in immunoelectrophoresis systems.

No differences between the soluble antigens of the three A. marginale isolates were detectable by agar gel diffusion. All three antigens moved to the same mobility zone in agar gel electrophoresis systems and each antigen formed an arc of precipitation when reacted with serum from calves infected with homologous or heterologous strains of A. marginale.

A beta and a gamma serum protein component, not exhibited in normal bovine serum, were present in the serums of animals infected with either of the virulent A. marginale strains or the attenuated strain.

DALEY, C. A.: A Sequential Study of the Pathogenesis of Disease Caused by Trypanosoma vivax in Experimentally Infected Calves, Utilizing Clinical, Pathological, Histopathological and Immunofluorescent Techniques. A Thesis submitted to the Graduate College of Texas A&M University in partial fulfillment of the requirement for the degree of Master of Science, May 1971.

Trypanosoma vivax obtained from a clinically sick cow near Neiva, Colombia, was passed in a sheep and a calf and inoculated into the jugular vein of 14 Holstein-Friesian calves. Fever occurred by 24 hours, and recurring parasitemia commenced after 72 hours. Associated with the first and subsequent parasitemias were decreases in hemoglobin, PCV, M:E ration, serum albumin, A:G ratio and neutropenia.

All calves exhibited gradual weight loss by 2 weeks and later submandibular edema usually became evident. Consistent post mortem lesions seen after 4 weeks were conspicuously hypertrophied, edematous lymph nodes, hypertrophied hemal lymph nodes, emaciation, rounded right heart, palpably firm liver, atrophied thymus and hypertrophied femoral bone marrow.

Associated with T. vivax of the infecting inoculum and succeeding parasitemias were generalized endothelial hypertrophy and mononuclear cell infiltration along blood and lymph vessels with proteinuria and bone marrow hyperplasia. At 3 weeks there were aggregations of macrophages containing engulfed material distributed along capillaries in pulmonary interalveolar tissue, and this lesion in combination with the anemia and apparent cardiac insufficiency were thought important in the development of anoxia, and probably contributed to the single fatality observed.

BISHOP, J. P.: Immune Response of Cattle Inoculated With Irradiated Babesia bigemina. A Dissertation submitted to the Graduate College of Texas A&M in partial fulfillment of the requirement for the degree of Doctor of Philosophy, December 1971.

Babesia bigemina parasitized blood exposed to varied doses gamma radiation up to 60 kRad was inoculated into calves. Calves infected with  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes exposed to doses up to and including 30 kRad developed progressive parasitemias. Some calves receiving  $1 \times 10^{10}$  parasitized erythrocytes irradiated at levels of 36 and 42 kRad did not develop progressive infections. Progressive infections were prevented by exposure to irradiation at 48 kRad or higher. Subinoculations into susceptible splenectomized calves from parasites thus treated failed to produce active infections.

A degree of acquired resistance to infection with B. bigemina developed in calves after 1 inoculation with B. bigemina parasitized blood irradiated at 48 and 60 kRad. The resistance was sufficient to suppress multiplication of the Babesia and to permit calves to survive otherwise severe clinical infections with nonirradiated parasites. There was also less erythrocytic destruction and a smaller increase in rectal temperatures following challenge. Presumably, the irradiated parasites were responsible for the development of resistance since irradiated nonparasitized blood did not produce a discernable acquired resistance.

The acquired resistance to infection with B. bigemina developed in calves inoculated with  $1 \times 10^{10}$  B. bigemina irradiated at 48 and 60 kRad was similar to the acquired resistance developed in calves inoculated with  $1 \times 10^{10}$  nonirradiated B. bigemina. It seems likely that the protective immunity produced with irradiated B. bigemina may be similar to that produced with living pathogenic B. bigemina in non-fatal infections. The acquired resistance to infection with B. bigemina developed in calves inoculated with  $1 \times 10^{10}$  B. bigemina irradiated at 48 and 60 kRad was much greater than the acquired resistance to infection developed in calves inoculated with  $1 \times 10^{10}$  heat killed B. bigemina. Thus, it seems likely that immunization with irradiated Babesia may provide the special immunological properties of living parasites important for producing a strong immunity while suppressing the pathogenic effects of the parasite. The Babesia parasites could be irradiated and frozen without apparent loss of immunizing properties.

TODOROVIC, R. A.: Babesiellosis Bovina en Australia. Revista de la Facultad de Medicina Veterinaria y de Zootecnia. Vol. 32, No. 1 & 2, 45-59, 1970.

Bovine babesiosis is still of great importance as a threat to the livestock industry in Australia. Due to the complexity of the epidemiology of this disease and other factors, the eradication of this hemoprotzoan malady is not possible at the present time.

The Commonwealth Scientific and Industrial Research Organization (CSIRO) is actively engaged in control and research on Babesia. Other research and teaching institutions, involved in the same problem include - the University of Queensland; New South Wales, Department of Agriculture, Cattle Tick Research Station; Queensland State Department and Animal Health Station. All of these research projects on Babesia are sponsored mainly from the Government of Australia.

The Australian research workers have contributed more than a hundred scientific publications on the various areas of Babesia research; they are foremost in this field, and the best trained in the world. The research laboratories are equipped with modern scientific tools, and staffed with well-trained technicians who successfully operate these instruments. The facilities are excellent and designed particularly for Babesia research. (Slides of these facilities are available for those who are interested).

The experience from this visit and knowledge obtained through discussion with Australian scientists working on different research projects will be invaluable for organizing a similar research program on Babesia in Colombia, South America. Furthermore, the Australian scientists with whom I visited all realized the importance of our mission in South America and expressed their willingness to cooperate with us in any manner in the future. They will be able to come to Colombia and spend time on short or long term assignments if funds are available.

CARSON, C. A., ADAMS, L. G., TODOROVIC, R. A.: An Antigenic and Serologic Comparison of Two Virulent Strains and an Attenuated Strain of Anaplasma marginale. Am. J. Vet. Res., Vol. 31, No. 6, 1071-1078, June 1970.

Soluble antigens of 3 Anaplasma marginale strains were compared by agar gel diffusion and immunoelectrophoretic techniques. Serum proteins from calves infected with each of the 3 A. marginale strains were separated electrophoretically and tested with rabbit anti-bovine serum in immunoelectrophoretic systems. There was no detectable difference between the soluble antigens or the 3 A. marginale strains. A beta globulin arc, which was not detectable in normal bovine serum was present in serum of acutely affected calves, and a gamma globulin arc was lengthened in the latter serum as compared with that in serum of normal calves.

GONZALEZ, E. F., TODOROVIC, R. A., ADAMS, L. G.: Ultraestructura de la Babesia bigemina. Revista ICA, Vol. 6, 89-112, No. 1, March 1971.

The morphology and some aspects related to the reproductive and feeding mechanism of Babesia bigemina have been studied by means of electron microscopy.

Although there are reports in the literature of the fine structure of Babesia canis which affects dogs, Babesia caballi which affects horses, and Babesia rodhaini which affects rodents, there is no report on the ultrastructure of B. bigemina which infects cattle.

B. bigemina was isolated from naturally infected cattle in the Valle del Cauca, Colombia and maintained in splenectomized calves in the Laboratorio de Investigaciones Medicas Veterinarias in Bogota. Blood samples were collected from the splenectomized animals at a time when the percentage of parasitized erythrocytes was 25%, and these samples were used for electron microscopic studies.

By means of the electron microscope different stages of B. bigemina were revealed such as oval, conoid and most commonly, pear shaped. The sizes of these forms were 2.5 to 6.5 microns in length by 2.3 microns in width. The young forms of the parasite were 1.5 by 2.5 microns. All these forms of parasites are surrounded by a dense cytoplasmic membrane which contained endoplasmic reticulum in the form of vesicles; these vesicles are composed of granules of different density. The endoplasmic reticulum appears as a homogenous mass with transparent vacuolar structures which are oval and spherical in shape. In addition to the endoplasmic reticulum, well defined dense polar bodies were found which appeared as oval shaped organelles, which communicated with the conoid part of the parasite by canals. The nucleus is the largest internal structure of the parasite and occupies one fourth to one third of its body. The nucleus is surrounded by a single membrane. Nucleoli were not revealed by electron microscopy.

Reproduction of B. bigemina appears to be carried out in two ways; by budding and binary fission. On the basis of these observations it is not clear which means of reproduction is more predominant. It is possible that both forms take place at the same time.

The feeding mechanism is not apparent. It appears that polar bodies play some role in this mechanism. These polar bodies could assume the function of food reservoirs of the parasite. It was also revealed that food vacuoles are similar to those in malarial parasites. The formation of food vacuoles probably results from an end process of pinocytosis as was described for Plasmodium species. We believe that both processes are involved in the feeding mechanism of Babesia parasites. Results of this study confirm the previously reported observation that there is no formation of pigment granules in Babesia; this implies that digestion of the host hemoglobin is complete; in contrast malarial parasites form hemozoin, a blood pigment, as an end product of metabolism.

TODOROVIC, R. A., GONZALEZ, E. F., AND ADAMS, L. G.: Immune Response of Cattle Vaccinated Against Babesiosis in Colombia, South America. Scientific Proceedings of the American Veterinary Medical Association and American Association of Veterinary Parasitologists (Las Vegas, Nevada, June 22, 1970).

Attempts to produce co-infectious and sterile immunity in cattle against Babesia infections have been carried out by vaccinating animals with live or killed-Babesia vaccines at Palmira, Valle del Cauca, Colombia (Altitude 1000 meters). Immune responses of the vaccinated animals were evaluated by several immuno-serologic methods. The degree of resistance to tick-borne challenge (Boophilus microplus naturally infected with Babesia spp) was determined by the percentage of recovery to normal parameters used in this study.

According to the experimental design used, a total of 110 animals were divided in 5 experimental groups to ascertain the immunologic responses. The first group consisted of 20 male, 85 kg., Holstein, 3 month-old calves which were premunized with Babesia bigemina, Babesia argentina, and 4 weeks later were exposed to tick-borne (Boophilus microplus) challenge. The second group consisted of 20 male, 95 kg., Holstein, 4 month-old calves subdivided into 4 groups and vaccinated with a killed-Babesia vaccine derived from the erythrocytes and plasma, respectively, of animals acutely infected with Babesia bigemina and Babesia argentina. The animals were inoculated with vaccine with or without Bacto-Adjuvant Complete H 37 Ra. The third group of 40 male, 80 kg., Holstein, 3 month-old calves was divided into two sub-groups. The first sub-group consisted of 20 animals which were premunized with Babesia bigemina and Babesia argentina and 8 days later were treated with a new experimental babesiacidal drug. The second sub-group which consisted of 20 animals was simultaneously premunized with Babesia spp. and Anaplasma marginale and later treated with their respective specific drugs. The fourth group consisted of 20 female, 75 kg., Holstein, 3 month-old calves prophylactically treated with drug No. 4A65 and 3 weeks later exposed to Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina. The fifth group consisted of 10 animals used as controls. Responses to vaccination and tick-borne challenge were evaluated by packed cell volumes, percentage of parasitemia, body temperatures, body weight, complement fixing antibody, titers, general physical conditions and percent recoveries after tick-borne challenge. Results in general indicate that resistance to babesiosis can be produced by co-infectious or sterile immunity. Experiments in prophylaxis, based on residual action of the babesiacidal drug, have given consistent and satisfactory results. In the future, it may be possible to develop control programs against bovine babesiosis based on these observations. The present status of these studies were described.

TODOROVIC, R. A., ADAMS, L. G., AND ROBERTS, D. E.: A Study of Bovine Babesiosis in Colombia, South America. Sci. Proc. 106th Annual Meetings, Minneapolis, Minn. Journal Am. Vet. Med. Ass., 154, 1399, 1969.

Our research program on bovine babesiosis is a part of the Institute of Tropical Veterinary Medicine, College of Veterinary Medicine, Texas A&M University, with the research program being sponsored by the Rockefeller Foundation and conducted at Laboratorio de Investigaciones Medicas Veterinarias laboratories, Bogota, Colombia, in cooperation with the Instituto Colombiano Agropecuario. This research effort is directed mainly toward the study and control of bovine babesiosis and the training of Colombian veterinarians and graduate students involved in these research projects.

Although bovine babesiosis is eradicated in the United States, the disease still occurs in most of the world and is of great importance as a threat to livestock industry, especially in the tropical areas of Latin American countries. In Colombia, babesiosis was first described by Lleras (1908) and later recognized as a widely distributed disease, causing great losses in purebred dairy cattle imported into enzootic areas. At the present time the incidence of babesiosis in Colombia is difficult to estimate. The disease exists as a mixed infection of Babesia bigemina, Babesia argentina and Babesia major, and the incidence of infection appears to be related to the occurrence and activity of the tick-vectors at the various altitudes.

The experiments were carried out to identify the existing Babesia species occurring in Colombia by morphologic, immunoserologic, pathologic, and chemotherapeutic methods. The immunoserologic relationship of Babesia spp. and strains were studied by gel-double diffusion precipitation, immunoelectrophoresis and fluorescent antibody techniques. Attempts were made to develop a sensitive and practical serologic test for the diagnosis of the latent Babesia infection. Several groups of intact and splenectomized calves were inoculated with various antigens isolated from the blood of cattle with acute babesiosis, and the blood from patent carriers, respectively. Response to vaccination, premunition, and challenge by tick-borne Babesia were recorded. The results of these experiments were discussed.

ADAMS, L. G., AND TODOROVIC, R. A.: A Study of the Pathogenesis of Anaplasmosis in Intact Calves: Including Clinical, Clinical Pathological, Serological and Immunofluorescent Techniques. Sci. Proc. VI Congreso Panamericano de Medicina Veterinaria y Zootecnia. Santiago de Chile, Sept. 28 - Oct. 3, 1970, 37.

Twelve 4-month-old male, hemotropic disease-free, Holstein calves were inoculated subcutaneously with blood containing a Colombian isolate of Anaplasma marginale. Previous to inoculation 3 control samples were taken for bone marrow and blood determination.

Thereafter, samples were collected every 2 days and one calf was euthanized every 2 days to collect a complete set of tissues for gross and microscopic pathological lesions as well as for the immunofluorescent study using the indirect technique. Results obtained are discussed, except those related to immunofluorescent study.

TODOROVIC, R. A., AND ADAMS, L. G.: Serologic Diagnosis of Babesiosis  
Sci. Proc. XIX World Veterinary Congress, Mexico City, August 15-22,  
1971, 3, 1114-1116.

The detection of the carrier state of bovine babesiosis has presented a particularly difficult problem, because the blood from a high percentage of carrier animals does not contain sufficient Babesia parasites on which to base the diagnosis. Therefore, a great deal of past interest was concerned with the development of serologic techniques which would aid in diagnosing babesiosis.

In this review an attempt has been made to summarize and discuss the recent advances on sero-diagnosis of babesiosis in infected cattle with special attention to the serologic procedures used in Laboratorio de Investigaciones Medicas Veterinarias located in Bogota, Colombia. In the last two decades fundamental knowledges concerning the immuno-serology of several Babesia spp. has led to the development of sero-diagnostic procedures for detection of Babesia antibodies. The antigens used in these techniques originated from parasitized erythrocytes and serum or plasma of animals with acute babesiosis, and they were applied in several serologic tests. The complement-fixation reaction constituted one of the earliest tests for the diagnosis of babesiosis. In recent years considerable progress was made to improve the complement-fixation test for the diagnosis of babesiosis. In addition gel precipitation, fluorescent antibody, and agglutination techniques were applied for the detection of specific Babesia antibodies utilizing antigens from the parasitized erythrocytes as well as acute serum.

The investigations described in this report were conducted to develop the new techniques and to evaluate existing techniques for diagnosing bovine babesiosis. Research was executed in collaboration with the Instituto Colombiano Agropecuario in the Laboratorio de Investigaciones Medicas Veterinarias in Bogota, Colombia. Antigens of Babesia spp. were isolated by means of two techniques and used in the complement-fixation test for the detection of Babesia antibodies in cattle experimentally and naturally infected. By means of the complement-fixation test it was possible to detect specific antibodies in the serum of cattle 8 days after blood-borne infection. A total of 5,420 serum samples of cattle infected with babesiosis were tested. The cattle were from several Colombian experimental herds with known histories of babesiosis located in Valle del Cauca, Rio Magdalena, Llanos and Monteria and from cattle artificially infected in the Laboratorio de Investigaciones Medicas Veterinarias in Bogota. Approximately 95% of these samples were positive whereas about 5% gave discordant reactions. In addition to the complement-fixation test used in our laboratory, attempts were made to apply the double-gel diffusion for characterization of Babesia spp. antigen-antibody reactions. A cross reaction was noted between Babesia bigemina and Babesia argentina in this system. The application of latex-agglutination and hemoagglutination tests for the detection of the Babesia antibodies are still under investigation in our laboratory. As a result of these investigations and observations, it is apparent that more investigation is needed for the development of a practical serologic technique for the diagnosis of babesiosis and to help solve this complex biological disease problem in tropical and sub-tropical areas of the world.

TODOROVIC, R. A., GONZALEZ, E. MATEUS, G., AND ADAMS, L. G.: Simultaneous control of Helminths, Anaplasmosis and Babesiosis in Cattle. Revista de la Facultad de Medicina Veterinaria y Zootecnia, Bogota. (In Press).

A group of 50 male Holstein-Friesian calves, 3 to 4 months old were used to evaluate a control program for gastrointestinal and hemotropic parasites. The experiment was conducted at the ICA experimental station in Palmira, Valle del Cauca at an elevation of 1,000 m. The animals were divided into 3 groups.

Twenty animals were premunized against anaplasmosis and babesiosis simultaneously; 8 days later they were treated against babesiosis using the compound 4A65 at a dosage of 1 mg/kg of body weight, and 21 and 56 days after premunition they were treated intravenously with the compound 356-C-61 (5 mg/kg IV) against anaplasmosis.

Twenty animals were premunized against anaplasmosis as it was done with the animals in Group I. Animals in this group were vaccinated with AGS plus adjuvant vaccine against babesiosis. The vaccine was repeated 14 days later. Animals in Group I and II were treated twice during the experiment with Ripercol (Tetramisol) against gastrointestinal parasites.

Ten animals were not treated and were used as controls.

All three groups of calves were kept under the same environmental conditions and the same management. The experiment was carried out during a period of 8 months. Blood samples were collected to evaluate anemia and parasitemia. The antibody titer was determined by the complement-fixation test. The body weights were measured and the fecal samples were examined for the presence of gastrointestinal parasites. Animals in Groups I and II had a high degree of resistance to babesiosis and anaplasmosis infections as a result of effective premunition and vaccination techniques. However, the animals in the control group had clinical babesiosis and anaplasmosis and high infestation with gastrointestinal parasites.

The importance of simultaneous control of gastrointestinal and hemotropic parasites is pointed out and methods to control these parasites are given.

ADAMS, L. G., HIPOLITO, OSMANE, MORALES, HERNAN, GONGORA SANTOS AND JONES, LARRY P.: Dermatofilosis Bovina (Estreptotricosis cutanea) en Colombia. Revista ICA, Vol. V., No. 1, March 1970, 3-16.

Four cases of bovine dermatophilosis were diagnosed in Cordoba, Colombia and confirmed by bacteriological culture methods. Macroscopic and microscopic descriptions were made of the lesions caused by Dermatophilus congolensis.

TODOROVIC, R. A., ADAMS, L. G., VIZCAINO, O., AND GONZALEZ, E.: Research and Control of Bovine Babesiosis in Colombia. Sci. Proc. VI Congreso Panamericano de Medicina Veterinaria y Zootecnia, Santiago de Chile, September 18 - October 3, 1970, p. 36.

Research was carried out to develop an effective program for the control of bovine babesiosis in Colombia.

Experiments were carried out at the Palmira Instituto Colombiano Agropecuario (ICA) experimental station in Valle del Cauca, (Altitude 1,000 meters) to produce co-infectious and sterile immunity against bovine babesiosis. Calves randomly selected were divided into four groups according to the experimental design used to evaluate the immunoserological responses to vaccination against babesiosis and tick-borne challenge. The degree of this immunity was determined by tick and blood-borne challenge. The percentage of parasitemia (P), body temperature (T), percentage of mortality (M) were used as the basis for comparing the reaction produced after vaccination and challenge. Experiments were conducted to evaluate the prophylaxis, therapy, effects, dosage, route of infection, toxicity and response of the animals injected with a new Burroughs-Wellcome babesiacidal drug No. 4A65.

On the basis of the observations made from these experiments, conclusions can be drawn that some degree of sterile immunity exists, besides the well known co-infectious (premunition) immunity in Babesia infections. To understand the exact mechanism of this type of immunity, more work needs to be done. The degree of resistance and the duration of immunity in relationship to different environmental conditions, strains differences, and the pathogenicity of the Babesia spp. and the quality of tick-borne challenge need to be determined.

ADAMS, L. G., AND FERREIRA, W. L.: Necrobacillosis Neo-Natal en Ovinos. Revista ICA, Vol. 6, No. 3, September 1971.

Five cases of ovine neo-natal necrobacillosis, in the Sabana of Bogota, were diagnosed in lambs less than 2 weeks of age. Macroscopic and microscopic lesions were described and the diagnosis was confirmed by bacteriological cultural techniques. This report constitutes the first known notice of the disease in neo-natal lambs in Colombia.

TODOROVIC, R. A., LUQUE, G. F., AND ADAMS, L. G.: Contribution to the Study of the Tick Distribution in Colombia, South America. Revista de la Facultad de Medicina Veterinaria y Zootecnia (Accepted for Publication).

The purpose of this work was to collect and identify tick species involved in the epizootiology of bovine babesiosis in Colombia. Bovine babesiosis was reported in Colombia in 1888 but there is not any published evidence about tick species involved in the transmission of the disease. Although, Boophilus microplus is the predominant tick in medium and hot climates, the exact distribution of the tick in the different regions of Colombia is not known. To develop an effective control program, the distribution of tick species needs to be determined. This is the first attempt to obtain this information about tick distribution in Colombia.

Animals naturally infected with Babesia bigemina and Babesia argentina were used as a tick collection source. The infectivity of these animals was determined by blood smears and complement-fixation techniques. The animals were located on farms in Palmira (Valle del Cauca) and Turipana (North Coast), Magdalena River and Sumapaz River. Ticks were collected from different breeds Holstein-Freisian, Zebu, Blanco Orejinegro, and Costeno con Cuernos, in animals of different ages. The ticks were collected from different parts of the animal bodies and preserved in Ethanol 95%. Adults, nymphs and larvae were collected from both sexes for identification purposes during a 12 month period (January - December).

Dermacentor nitens was found in the animals infected with babesiosis in the Valle del Cauca, North Coast, Sumapaz River; Amblyomma cayennense was found in animals infected with babesiosis and anaplasmosis in the Magdalena River and the North Coast; Boophilus microplus was found in the same animals infected with babesiosis and anaplasmosis used in this experiment. Until the present time the significance of the findings of Amblyomma and Dermacentor ticks in epidemiology of babesiosis is not clear.

Experiments are in progress to determine the population and distribution of the tick species in other parts of Colombia for the purpose of investigating the exact role of Dermacentor nitens and Amblyomma cayennense in the transmission of bovine babesiosis.

MULLENAUX, CHARLES H., AND ADAMS, I. G.: La Oncocercosis Equina Asociada con el Mal de la Cruz en Colombia: Descripción de dos casos. Revista ICA, Vol. 6, No. 3, September 1971.

Two cases of equine fistulous withers were diagnosed in which Onchocerca spp. was found to be present in the affected tissues. One of the horses had a brucellosis antibody titer of 1:50 using the rapid plate agglutination method and, in the same animal, Brucella spp. was cultured from the suppurative materials of the nuchal bursitis of the withers. Macroscopic and microscopic pathological lesions caused by the nematode Onchocerca spp. were described.

ADAMS, L. G., CRAIG, THOMAS M., PLATT, K. B., AND WYSS, JOHN H.: Bovine Eperythrozoonosis in Colombia. Accepted by Revista ICA for publication. (In Press).

Eperythrozoon wenyoni, E. tejanodes and E. tuomii were diagnosed in 14 splenectomized Holstein-Friesian 4 to 11-month-old calves that originated from the Sabana de Bogota. Eleven calves had pure infections of E. wenyoni, 2 calves had dual infections of E. wenyoni and E. tejanodes, and one calf had a pure infection of E. tuomii. The diagnosis was determined on Giemsa-stained blood smears, and morphological descriptions of the Eperythrozoon spp. were given. Six splenectomized calves exhibited depression and anorexia, but all 14 calves had elevated rectal temperatures. Two calves had serious conjunctivitis with excessive lacrimation. The increase in rectal temperature coincided with the onset of parasitemia while the packed cell volume decreased after the onset of parasitemia. The average incubation period and standard deviation was  $14.9 \pm 3.5$  days post-splenectomy. Treatment with 2-di-(Beta, gamma-dioxipropil)-(aminofenol)-(4 arseno 5)-Beta-(Benzaxozalil)-(2)-mercaptol-propionato de sodio at 29 mg/kg intramuscularly caused the parasitemia to become undemonstrable within 24 hours with further recrudescence occurring within 6 weeks. This is the first report of bovine eperythrozoonosis due to Eperythrozoon wenyoni, E. tejanodes and E. tuomii in Colombia.

ADAMS, L. G.: Epizootia Espontanea de Hepatitis Toxica en Porcinos atribuida a Aflatoxicosis. Accepted by Revista ICA for publication. (In Press)

Nine of the 56, 4 to 6-month-old Duroc male and female pigs died 2 months after consuming a ration consisting of 8.75% moldy peanut meal. The pigs exhibited weight loss, roughened hair coat, anorexia, lethargy, icterus, melena, increased followed by decreased rectal temperature and death. The livers of the remaining 45 pigs were condemned due to cirrhosis. Serum sorbitol deshydrogenase activities, glutamic-oxaloacetic transaminase activities, bilirubin concentrations, serum beta globulin levels, serum gamma globulin levels and total serum protein concentrations were increased as serum albumin/globulin rations, albumin levels, packed cell volume and hemoglobin contents were decreased. No changes were observed in total leukocyte counts or serum alpha globulin levels.

The principal macroscopic lesions consisted of generalized icterus, petechial and ecchymotic hemorrhages with yellow transudates occurring in the body activities. Subendocardial as well as subserosal ecchymotic hemorrhage were commonly observed. Ulceration of the gastric fundus occurred which filled the stomach, duodenum, jejunum, ileum and colon with free digested and undigested blood. The liver was pale yellowish-brown, firm (increased cutting resistance) and cirrhotic with very accentuated hepatic lobules outlined by translucent bands. Hundreds of irregular round yellow to brown foci of hepatic nodular regeneration were interspersed throughout the hepatic parenchyma. The gall bladder was moderately edematous and contained a small amount of light green bile. The principal microscopic lesions of the liver were disorganization of the hepatic architecture, acinus formation, severe sinusoidal fibrosis, mild biliary hyperplasia, advanced hepatic nodular regeneration, extensive hepatocellular megalocytosis, hepatocellular anisocytosis, mild hepatocellular necrosis, fatty metamorphosis and moderate cholangiolar bile plug formation. The diagnosis and etiology of these 4 cases of porcine chronic toxic hepatitis was attributed to aflatoxicosis apparently produced by Aspergillus flavus growing on peanut meal. The present article is the first report of aflatoxicosis in Colombia.

HERNANDEZ, J. D., ROBERTS, E. D., ADAMS, L. G., AND VERA, T.: Pathogenesis of Hepatic Granulomas in Turkeys Infected with Streptococcus faecalis var liquefaciens. Avian Diseases, Vol. 16, No. 2, January - March 1972, 201-216.

The pathogenesis of hepatic granulomas in turkeys has been studied by reproducing the lesions experimentally with Streptococcus faecalis var. liquefaciens isolated during a field outbreak of turkey hepatic granulomas in Colombia. The 170 turkey poults (Bronze) used were 4 weeks old. Groups of poults were inoculated intravenously or orally with 0.1 ml. of a 24-hour culture of Streptococcus faecalis var. liquefaciens at a dilution of 3 x 10 on the MacFarland Nephelometer Standard 10. The oral route of inoculation reproduced a disease most similar to the naturally occurring disease.

Clinically, the acute phase of infection was characterized by a high mortality rate in the first to seventh days but only sporadically thereafter. The septicemic phase produced the formation of septic thrombi which localized in various organs, producing infarction with heterophilic infiltration. Once the septicemic phase of the problem passed, the disease was manifested primarily by a focal hepatitis initiated primarily as a focal necrotic cholangial lesion. The biliary epithelium had hyperplastic to degenerative processes which participated in the formation of biliary thrombi. Granulomas were characterized by focal areas of necrosis surrounded by Langhans-type giant cells, and macrophages.

**APPENDIX II**

## APPENDIX II

### The Relationship of Disease to Productivity

From an animal health viewpoint, hemotropic diseases represent the greatest single deterrent to meat and milk production in the tropics. These diseases are transmitted by arthropods such as tsetse fly, ticks, and mosquitoes. They are usually responsible for severe to moderate anemias, which are associated with weight losses, drops in milk production, and in cases of acute infection, death. Local indigenous cattle are thought to be resistant, in that deaths are not a common occurrence. Field and laboratory investigations in recent years confirm the relatively low mortality among indigenous cattle, but show an extremely high incidence of infection, accompanied by anemia, lack of growth, and low milk production. Frequently under these conditions the sick animals are not individually identified, since nearly all are infected. The resulting poor growth rate, and low milk output is then tolerated as being normal for the area. To a degree this may be true, but a cursory review of history shows that animal production can be greatly increased by the elimination of hemotropic infections. Prior to 1920 the Texas cattle industry was severely hampered by Babesia bigemina, which prevented the successful introduction of clean - genetically improved breeds into the area, and reduced the growth rates and production of indigenous cattle. These conditions were similar to those currently existing in Colombia. The elimination of this disease was accompanied by a rapid increase in beef and milk production.

The solution of the problem of babesiosis in Texas was facilitated when research showed that a single host tick, Boophilus annulatus, was responsible for its spread. The problem is compounded in many tropical countries, Colombia

being a good example, by multiple tick vectors, and combined infections caused by Anaplasma, Babesia bigemina, B. argentina, B. major and Trypanosoma. Notwithstanding these problems, recent research has revealed several approaches which offer the hope for more effective control and even possible eradication.

The economics of these diseases, or accurate calculations as to the dollar value of losses from these infections is difficult, but estimates are possible from available information. Dr. W. T. Oglesby (Louisiana State University) in 1962, as a result of a survey in the U.S., reported the probable losses due to anaplasmosis in the 16 states where this condition is a problem. He estimated that in 1958 deaths due to anaplasmosis were responsible for a \$12,000,000.00 loss, with another \$36,000,000.00 loss being associated with weight reduction, lowered productivity, increased feed costs, etc. for a total loss of \$48,000,000.00 (Proceedings of the 4th National Anaplasmosis Conference, April 1962). There are approximately 31,500,000 cattle in those states where anaplasmosis is most common. On the basis of a 16,232,697 cattle population in Colombia (Departamento Administrativo de Estadística) it may be expected that an approximate \$25,000,000.00 annual loss would be incurred if cattle values were equivalent. Assuming cattle values to be 66% of those in the U.S. then a loss of 16.5 million dollars might be expected annually in Colombia. This figure is really only a small part of the annual losses, when we consider babesiosis and trypanosomiasis. Based on experimental results obtained at Turipana (1971-72) losses from Babesia and Anaplasma for Colombia have been estimated to be at least 25.0 million dollars or 56 million pounds of beef. These diseases are endemic throughout Latin America, therefore this figure should be multiplied many times to acquire a true picture of actual losses. Perhaps of greater concern than the actual monetary losses, which these diseases cause, is the lack of productivity, the failure to achieve the

full potential for which the land is capable, resulting in an absence of food and animal protein which are irreplaceable, and can only aggravate the already serious shortages which exist in the world. Control of the arthropod born hemotropic diseases is an essential pre-requisite to achieve the full measure of livestock productivity in tropical areas of the world.

Contributions by Colombia to This Program:

The seriousness of this problem is generally recognized throughout Latin America, and particularly so in Colombia. Colombian government agencies, have contributed generously to the support of our program since its beginning in 1967. Until 1971 our staff and graduate students were housed at the LIMV laboratories of The Instituto Colombiano Agropecuario (ICA) in Bogota. During this period we received assistance in the acquisition and maintenance of laboratory animals, and also the use of laboratory space and facilities, at no cost to our project. In early 1971 a new veterinary laboratory at Turipana, on the North coast near Monteria, was completed, primarily for the study of hemotropic diseases. This laboratory is headed by a former graduate student at Texas A&M. Approximately 80 cattle furnished by the ICA-TURIPANA Station, are presently on trial using several control approaches based mainly on premunition.

The facilities available to us at Turipana are ideal for field testing new methods of control. The cooperation and assistance of local authorities has been excellent, and shows every indication of continuing.

In 1971 our staff moved to the new Centro Internacional de Agricultura Tropical (CIAT) laboratories at Cali. The Colombian government made land available for CIAT, and has a close relationship with this organization which cooperates with ICA.

Research Accomplishments:

- (1) Premunization with virulent Anaplasma and Babesia in indigenous

cattle maintained under lowland, tropical conditions has proven effective in significantly reducing mortality and production losses. The initial animal response to these virulent organisms was modified by specific chemo-therapy.

- (2) A new vaccine for anaplasmosis has been tested in Colombia and offers some promise despite initial setbacks. This vaccine preimmunizes cattle with practically no adverse reaction. Preimmunization of young calves with virulent organism can produce a persistent anemia for up to 50 days whereas the vaccine produces a milder response and an anemia that persists less than 8 days.
- (3) A new therapeutic process has resulted in preimmunization with a virulent organism with no drop in PCV. This immunity has persisted over 4 months with no signs of clinical disease.
- (4) A therapeutic approach has been developed which will completely eliminate infection with anaplasmosis with only 3 injections which is 4 times more effective than previous methods.
- (5) Work is in progress for improved killed vaccines for both Babesia and Anaplasma.
- (6) Since the inception of The Institute of Tropical Veterinary Medicine, over 44 scientific papers have been published. This plus unpublished research data is forming the basis of current and future control programs for the containment of these costly diseases.
- (7) Like the U.S., some areas of Colombia are free of these diseases. A survey has been completed which identifies problem areas, and establishes very definite epidemiologic patterns of infection.
- (8) Progress has been accelerated by the discovery of 2 new drugs

a dithiosemicarbazone (356 C 61) and Imidocarb (4A65) which show much greater activity as therapeutic agents.

- (9) Imidocarb has been demonstrated to have prolonged prophylactic value against Babesia.
- (10) Studies to develop a new vaccine using irradiated Babesia has shown a degree of success, and offers hope for a safer vaccine for use in preventing babesiosis.
- (11) Improved diagnostic procedures have been developed for the identification of Babesia.
- (12) Immunological studies have shown that a sterile immunity in Babesia can be induced in susceptible cattle, which is contrary to previously held theories that carrier status or co-infectious immunity was essential.
- (13) Babesia argentina and B. bigemina have been shown to be immunologically different. A B. argentina challenge of a B. bigemina carrier produces a more severe reaction than the opposite challenge.
- (14) Purified isolates have been made of A. marginale, B. bigemina, B. argentina, T. vivax, and T. evansi from cattle in Colombia.
- (15) Non-infected Boophilus microplus ticks have been selectively infected with pure isolates of B. argentina and B. bigemina.

Training or Teaching Accomplishments:

- (1) Three U.S. graduate students have completed their work for the M.S. degree and 1 has completed and received his Ph.D.
- (2) We now have 5 candidates for the M.S. degree and 2 candidates for the Ph.D. degree currently pursuing their work.
- (3) Seven Colombian graduate veterinarians both at Texas A&M and in Colombia have and are pursuing degrees and or training with a

major emphasis on hemotropic diseases.

- (4) The research programs plus the training of Colombian students has focused attention on these hemotropic diseases to the extent that more work is being done to control these diseases by Colombians in their own country.
- (5) In June 1971, Dr. R. A. Todorovic, went to Peru at the request of the USAID mission there, to aid in projecting an evaluation program for control of hemotropic diseases in cattle. This activity further emphasizes the point that much of the work done in Colombia will be applicable throughout Latin America.

Application of Research Findings:

A field program has been underway for over a year at Turipana putting into practice the scientific principles developed in the laboratory. Direct data from these experiments is not yet available, but it is known that when anaplasmosis and babesiosis are absent or controlled that productivity as measured by growth rates will increase.

The effects of moving clean adult dairy type stock from a hemotropic disease free area, such as the Bogota Savannah, to the North coast without adequate vaccination or treatment is invariably followed by drastic weight loss, an almost complete drop in milk production, and in many instances death within 60 days. Treatment can in most instances prevent death, but the persistent re-exposure pattern prevents economic milk production. Weight gains of 4-6 month old calves moved to Turipana showed the effects of hemotropic disease. The untreated controls showed an average gain of 36 pounds in the first 60 days, and for the next 60 days an average loss of 4 pounds for a net gain of 32 pounds. Among calves premunized with the attenuated vaccine an average gain of 52 pounds was recorded for a net increase of 20

pounds or 62% per calf. Among calves premunized with virulent Anaplasma an average weight gain of 56 pounds was recorded for a net increase of 30 pounds or 94% per calf.

If these figures are representative and we reflect this increased weight gain to a ranch weaning 1,000 calves we could expect 20-30,000 pounds more in live weight or 10-15,000 pounds more from essentially the same feed input.

The potential for increase in beef and milk production through the control of these hemotropic diseases is one which is well worth pursuing.

An increasing number of veterinarians and livestock producers, are utilizing the therapeutic, and immunologic approaches which have been demonstrated effective in preventing losses. Most of the research papers resulting from this work are published in Spanish for greater distribution in Colombia and South America.

A recent field trial, at Turipana (Colombia's North coast) has recently been completed. This trial demonstrated significant advantages to the use of vaccination and treatment procedures in indigenous calves, to prevent hemoparasite losses. Twenty calves, in which disease control was achieved, showed an 89% increase in weight gains during a 257 day observation period, when compared to untreated controls.

**APPENDIX III**

**Budget Statement for Past Year (1971-72)**

**Statement of Expenditures According to Major Work Goals**

	<b>Anaplasmosis 30%</b>	<b>Babesiosis 48%</b>	<b>Trypanosomiasis 20%</b>	<b>Theileriasis 2%</b>	<b>Total (100%)</b>
<b>Personnel Salary &amp; Allowances</b>	\$40,599.00	\$64,958.00	\$27,066.00	\$2,706.00	\$135,329.00
<b>Travel and Transportation</b>	11,828.00	18,925.00	7,885.00	788.00	39,426.00
<b>Equipment</b>	13,531.00	21,651.00	9,021.00	902.00	45,105.00
<b>Supplies and Operation</b>	23,201.00	37,121.00	15,467.00	1,547.00	77,336.00
<b>Overhead</b>	11,746.00	18,793.00	7,830.00	784.00	39,153.00
<b>Total</b>	100,905.00	161,448.00	67,269.00	6,727.00	336,349.00

**Budget Statement for the Coming Year (1972-73)**

**Statement of Expenditures According to Major Work Goals**

	<b>Anaplasmosis 30%</b>	<b>Babesiosis 48%</b>	<b>Trypanosomiasis 20%</b>	<b>Theileriasis 2%</b>	<b>Total (100%)</b>
<b>Personnel Salary &amp; Allowances</b>	\$54,947.00	\$87,914.00	\$36,631.00	\$3,663.00	\$183,155.00
<b>Travel and Transportation</b>	10,354.00	16,567.00	6,903.00	691.00	34,515.00
<b>Equipment</b>	1,650.00	2,640.00	1,100.00	110.00	5,500.00
<b>Supplies and Operation</b>	14,511.00	23,217.00	9,674.00	967.00	48,369.00
<b>Overhead</b>	10,038.00	16,062.00	6,692.00	669.00	33,461.00
<b>Total</b>	91,500.00	146,400.00	61,000.00	6,100.00	305,000.00

**PROGRESS REPORT OF HEMOTROPIC DISEASE RESEARCH**

**APPENDIX III**

**May, 1972**

**College of Veterinary Medicine**

**Texas A&M University**

**College Station, Texas**

Institute of Tropical Veterinary Medicine  
College of Veterinary Medicine  
Texas A&M University

May, 1972

Studies on Hemotropic Diseases

Progress Report

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The general administrative support and technical assistance provided by the College of Veterinary Medicine, and the various academic departments has been generous, and is greatly appreciated.

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Combined Treatment With a Dithiosemicarbazone and Oxytetracycline  
To Eliminate Anaplasma marginale Infections in Splenectomized Calves  
(Accepted for Publication in Research in Veterinary Science)

Introduction:

A recent proposal for the eradication of anaplasmosis (Wheeler, 1968) is based largely on the principle of identifying infected animals and then eliminating the infection by treatment. The need for effective therapeutic agents in such a program is obvious. Carrier cattle, while resistant to future Anaplasma exposure, represent a source of infection that can only be eliminated by treatment or slaughter.

Foote et al. (1951), Foote and Wulf (1952), and Splitter and Miller (1953), recognized the value of the tetracyclines in treating anaplasmosis, and reported success in eliminating infection with these agents. To eliminate infection by these drugs was often expensive and time consuming. Barrett et al. (1965) described the successful use of an alpha-dithiosemicarbazone (356 C 61)\* against a number of microorganisms including A. marginale. Brown et al. (1968) and Roby et al. (1968) have reported the specific activity of this compound in the treatment of anaplasmosis. Kuttler and Zaraza (1970) and Kuttler and Adams (1970) confirmed the value of 356 C 61 but they were unable to entirely eliminate infection at sub-toxic levels of the drug. A more recent study by Kuttler (1971) has shown that combined therapy with both oxytetracycline and 356 C 61 gave better results than either drug alone.

These trials report the use of 356 C 61 and oxytetracycline\*\* given simultaneously at differing levels, frequencies and numbers of injections

\* Gloxazone (Alpha Ethoxyethylglyoxal dithiosemicarbazone), Burroughs-Wellcome & Co., Inc. - Raleigh, North Carolina (experimental drug only).

\*\* Liquamycin injectable, Chas. Pfizer & Co., Inc. - New York, N.Y.

in an effort to find a regime which will effectively eliminate infection with the greatest economy of time and drug.

Materials and Methods:

A total of 36 splenectomized calves infected with Anaplasma marginale have been used to evaluate various treatment schedules. These calves were dairy type, predominantly Holstein breeding, and averaged  $10 \pm 2$  months of age. A total of 12 different treatment schedules were followed, using 2, 5, and 10 mg/kg 356 C 61, and 11, and 22 mg/kg oxytetracycline. All injections were made intravenously, diluting and mixing 356 C 61 and oxytetracycline with approximately 150 ml sterile physiological salt solution (0.85% NaCl). These drugs were given simultaneously in varying amounts; 1, 2 and 3 times at 24, 48, and 72 hour intervals. The 12 schedules of treatment are listed in Table 1.

Each calf was bled twice weekly for a minimum of 2 weeks before treatment, for at least 8 weeks after treatment, and once a week for at least another 8 weeks. Packed cell volumes (PCV), a complement-fixation (CF) tests for anaplasmosis, and Anaplasma parasitemia as determined on Giemsa-stained blood smears, were conducted on all blood samples.

The occurrence of 1% or greater Anaplasma parasitemia after treatment was considered evidence of a relapse infection and indicative of failure for the treatment procedure being tested.

Infectivity trials were conducted on all calves failing to show evidence of a relapse parasitemia an average of  $87 \pm 20$  days after treatment, by sub-inoculating 200 ml of blood I.V. into a susceptible splenectomized calf. On the 21 calves failing to show a relapse infection 17 were challenged an average of  $164 \pm 78$  days after treatment, by the inoculation of 5-10 ml of known A. marginale infected blood. A response to this challenge was a further indication of non-carrier or susceptible status in the treated calves.

Results:

Calf response to treatment is presented in Table 2. Observations 2 weeks after treatment showed marked increase in PCV and a disappearance of a demonstrable A. marginale parasitemia in all groups. With the exception of group VI a reduction in CF titer was observed. In group VI CF titer remained unchanged 2 weeks after treatment. Animals of this group lost their CF titers later in the course of the experiment.

Relapse infections occurred in all animals treated in groups I, II, VIII, IX, X, XI, and XII. Only 1 of 3 calves in group V showed a relapse. The range of relapse time occurring for all groups was 25 to 45 days, with an average of  $38 \pm 15$  days. No significant differences in relapse times could be demonstrated and with few exceptions there was surprisingly little variation in the relapse time.

All calves in groups III, IV, VI, and VII, treated with 5 or 10 mg/kg 356 C 61 and 11 mg/kg oxytetracycline given 3 times at 24 or 48 hour intervals, failed to show evidence of relapse infection. Two of 3 calves from group V, treated with 5 mg/kg 356 C 61 and 11 mg/kg oxytetracycline 3 times at 72 hour intervals failed to show signs of relapse. Infectivity trials on all 21 calves were negative. The loss of CF activity among these calves, recorded in Table 2, was highly variable as noted in the large standard deviations. All 17 calves checked for susceptibility by challenge with infected blood, responded by showing A. marginale parasitemias.

Discussion and Conclusions:

Interval, and the number of injections appear to be important factors in the success or failure of the treatment programs. Single or 2 treatment schedules were consistently unsuccessful, even though the amount of drug injected was equal to or greater than the successful programs involving 3 injections. All successful treatment involved the simultaneous I.V. injection

of 5 or 10 mg/kg 356 C 61 and 11 mg/kg oxytetracycline. No clear cut preference was obvious in comparing the 24 and 48 hour intervals. Less than 5 mg/kg 356 C 61 was ineffective even when combined with oxytetracycline. Treatment intervals of over 48 hours were less effective.

It is possible and probable that treatment data obtained from splenectomized calves is not always applicable to intact calves. These trials must be repeated in intact animals before the precise dose level, number of treatments and interval can be determined. Splenectomized calves present an obvious advantage in early screening trials due to the consistent relapse pattern occurring in those instances where treatment is ineffective. These reactions are easily recognized, thus eliminating the expense and time required to conduct infectivity checks on all animals. In every instance reported in these trials failure of a relapse to occur within 62 days was indicative of successful elimination of infection. A loss of CF response in calves successfully treated was a further indication of the elimination of infection, but this factor was extremely variable among individuals, even though actual infection had been removed.

Summary:

A total of 12 treatment schedules combining oxytetracycline and an alpha-dithiosemicarbazone (356 C 61) were tested on 36 splenectomized calves carrying Anaplasma marginale infections. Anaplasma infection was eliminated following the administration of 5 or 10 mg/kg 356 C 61 combined with 11 mg/kg oxytetracycline, and given 3 times at 24 or 48 hour intervals. Treatments employing lower drug levels, fewer injections, or at greater time intervals failed to eliminate infection.

Treated, splenectomized calves failing to show evidence of an A. marginale relapsing infection within 62 days were found to be free of infection

(5)

on the basis of infectivity trials conducted an average of 87 days after treatment, and by re-inoculation with A. marginale an average of 164 days after treatment.

Table 1

Quantity of Drug, Number of Injections, and Injection Interval  
Used to Treat A. marginale Infected Splenectomized Calves

Treatment Group	No. of Animals	Quantity of Drug Used		No. of Injections	Treatment Interval
		356 C 61 mg/kg	Oxytet. mg/kg		
I	2	2	11	3	24 hrs
II	1	2	11	3	48 hrs
III	3	5	11	3	24 hrs
IV	8	5	11	3	48 hrs
V	3	5	11	3	72 hrs
VI	3	10	11	3	24 hrs
VII	5	10	11	3	48 hrs
VIII	2	5	11	2	24 hrs
IX	2	5	11	2	48 hrs
X	4	10	11	2	48 hrs
XI	1	10	22	1	- -
XII	2	5	11	3	48 hrs

Oxytet: Oxytetracycline.

Table 2

The Effects of Treatment Administered to Splenectomized Calves  
Infected With Anaplasmosis

Treat. nt Group	No. of Calves	Pre-treatment			2 Weeks Post-treatment			No. To Relapse	Avg. Relapse Time Days	(1) Loss of CF Titer	(2) Infectivity Avg. No. of Days Post Treatment	(2) Trials Results
		PCV	Paras.	CF	PCV	Paras.	CF					
I	2	20.5 ± 3	4.0 ± 5.4	1:113	26.0 ± 1	0	1:40	2	25	--	--	--
II	1	14.0	0.2	1:80	29.0	0	1:40	1	45	--	--	--
III	3	11.0 ± 3	30.0 ± 32.0	1:80	25.3 ± 10	0	1:40	0	--	78 ± 33	114 ± 3	Neg.
IV	8	18.7 ± 4	1.0 ± 0.9	1:62	30.0 ± 2	0	1:22	0	--	58 ± 30	76 ± 16	Neg.
V	3	23.0 ± 3	0.2 ± 0.15	1:50	29.0 ± 12	0	1:8	1	39	50 ± 25	103 ± 0	Neg.
VI	3	16.7 ± 3	2.0 ± 2.0	1:32	24.7 ± 2	0	1:32	0	--	75 ± 53	60 ± 0	Neg.
VII	5	17.0 ± 3	0.8 ± 0.7	1:46	27.4 ± 2	0	1:8	0	--	35 ± 12	96 ± 9	Neg.
VIII	2	15.5 ± 5	0.2 ± 0.0	1:113	28.0 ± 4	0	1:56	2	35	--	--	--
IX	2	13.5 ± 5	19.0 ± 21.0	1:111	22.0 ± 4	0	1:80	2	42	--	--	--
X	4	17.2 ± 4	2.2 ± 0.5	1:56	29.0 ± 2	0	1:17	4	45	--	--	--
XI	1	11.0	2.0	1:160	27.0	0	1:80	1	35	--	--	--
XII	2	19.0 ± 6	1.0 ± 1.0	1:40	25.0 ± 0	0	1:8	2	31	--	--	--

(1) Days required for CF titer to drop below the diagnostic 1:5 titer.

(2) A total of 21 calves failing to relapse after treatment were tested for residual infectivity by subinoculating 200 ml, blood into splenectomized calves. This was done at varying times after treatment.

Paras.: Parasitemia or percent parasitized erythrocytes.

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Stillwater, Oklahoma, 1.

Promising Therapeutic Agents for the Elimination  
of Anaplasma marginale in the Carrier Animal

(Presented at the USAHA Meetings Oct. 1971 to be Published in Proceedings)

The need for and the implementation of an effective program for the reduction of losses due to anaplasmosis has been discussed for years by both the veterinarian and the livestock producer. The continuation of research progress inevitably brings the goal of anaplasmosis control nearer. In recent years significant progress has been made in the area of vaccine production (3, 11, 14), and in the development of new and effective diagnostic procedures (1). Both of these advances can perform an important function in the reduction of animal losses due to anaplasmosis. The persistent nature of Anaplasma infections, even after apparent recovery, creates serious problems when eradication programs are considered. A therapeutic approach which would effectively eliminate the disease agent from infected animals will probably play an important role in control programs of the future.

For many years the tetracyclines have been the drugs of choice in treating clinical anaplasmosis and in eliminating the carrier state of infection. A number of workers (4, 6, 7, 13) have in the past reported on the efficacy of these drugs showing them to be useful and effective. The elimination of the carrier status with tetracyclines usually requires either daily injections for 10 or more days or a low level feeding program for 30 to 60 days. Both procedures are time consuming and expensive. A more efficient regime to eliminate carrier infections would significantly contribute to the control of anaplasmosis.

A program of test and treatment appears practical for the eradication of anaplasmosis in some areas of the country (15). The problem of eradication is more difficult in those areas where ticks serve as biological vectors and wildlife are a reservoir of infection.

During the past few years a new drug, in addition to the tetracyclines, has attracted some attention as a possible therapeutic agent. In 1965 the effectiveness of an a-dithiosemicarbazone (356 C 61)\* in the treatment of anaplasmosis, was reported (2). Since that time several reports have been published confirming the effectiveness of this agent (5, 9, 10, 12). More recently favorable results have been reported using the a-dithiosemicarbazone with oxytetracycline\*\* (8). The results suggest that the simultaneous use of these drugs was more effective than either alone.

In addition to the dithiosemicarbazone a second material, Imidocarb (3,3'-Bis-(2-imidazolir 2-yl) - carbanilide dihydrochloride) or 4A65,\* has been tested and shows promise in the treatment of anaplasmosis.

This report will review results of studies dealing with these 3 agents, oxytetracycline, 356 C 61, and 4A65, when used for the elimination of anaplasmosis carrier status in splenectomized calves.

Splenectomized calves have proven useful in these preliminary studies largely because of the characteristic A. marginale relapses which occur in treated animals that retain a non-apparent infection. In these experiments, calves failing to show a relapse within 62 days of treatment have been shown to be free of infection by subsequent infectivity tests, and in many instances by later re-challenge. Splenectomized calves are an abnormal system, and these results should not stand alone without further trials in intact cattle.

\* Burroughs-Wellcome and Co., Inc., Raleigh, North Carolina.

\*\* Liquamycin injectable: Chas. Pfizer & Co., Inc., New York, N.Y.

These results are useful to delineate the relative activity of various treatment schedules, and provide the basis for future studies.

Materials and Methods:

Treatment was administered to 59 splenectomized, A. marginale-infected dairy type calves of mixed breeding ranging in age from 5-21 months with an average of  $10.8 \pm 4$  months.

Groups I, II, and III consisted of 14 calves treated with 356 C 61 only (Table 1). Calves in Group I were treated 5 times at 24 hour intervals with 5 mg/kg injected intravenously (I.V.). Calves in Group II were treated 10 times at 24 hour intervals with 5 mg/kg injected I.V. A total of 8 calves in Group III were injected intramuscularly (I.M.) either 3 or 4 times with 5 mg/kg 356 C 61 at varying intervals. Treatment was administered to 1 calf, 3 times, at 48 hour intervals; to 3 calves, 4 times, at 3 and 4 day intervals (twice a week); to 3 calves, 3 times, at 7 day intervals; and to 1 calf, 3 times, at 14 day intervals.

Groups IV, V, VI, VII, and VIII consisted of 33 calves treated with 356 C 61 and oxytetracycline (Table 2). Calves of Group IV were treated 3 times at either a 24 or 48 hour interval with 2 mg/kg 356 C 61 and 11 mg/kg oxytetracycline. Calves of Group V were treated 3 times at either a 24 or 48 hour interval with 5 mg/kg 356 C 61 and 11 mg/kg oxytetracycline. Calves of Group VI were treated 3 times at 72 hour intervals with 5 mg/kg 356 C 61 and 11 mg/kg oxytetracycline. Calves of Group VII were treated 3 times at either a 24 or 48 hour interval with 10 mg/kg 356 C 61 and 11 mg/kg oxytetracycline. Calves of Group VIII were treated 2 times at either a 24 or 48 hour interval with 5 or 10 mg/kg 356 C 61 and 11 mg/kg oxytetracycline. The 356 C 61 and oxytetracycline were mixed in and diluted with 150 ml 0.85% NaCl (PSS) and injected I.V.

Groups IX and X consisted of 12 calves tested with 4A65 alone and in

combination with 356 C 61 and oxytetracycline (Tables 3 and 3A). Calf 405 was treated 1 time with 4 mg/kg 4A65, 15 mg/kg 356 C 61 and 44 mg/kg oxytetracycline. The 4A65 was injected I.M., and the 356 C 61 and oxytetracycline were diluted in PSS and injected I.V.

Calves 135 and 219 were each treated 1 time, with 6 mg/kg 4A65 I.M., and 15 mg/kg 356 C 61 I.V. Calves 424 and 431 were each treated 1 time with 15 mg/kg 4A65 I.M. (Table 3).

Calves 217 and 248 were each treated 3 times at 24 hour intervals with 2 mg/kg 4A65 I.M. and 5 mg/kg 356 C 61 I.V. Calf 450 was treated 3 times at 24 hour intervals with 5 mg/kg 4A65 I.M., and 2 mg/kg 356 C 61 I.V. Calf 245 was treated 3 times at 24 hour intervals with 4 mg/kg 4A65 I.M. Calves 405 and 413 were each treated 3 times at 24 hour intervals with 5 mg/kg 4A65 I.M. Calf 265 was treated 3 times at 24 hour intervals with 6 mg/kg 4A65 I.M. (Table 3A).

All animals were monitored for changes in percent parasitemia, packed cell volume (PCV), and serum complement-fixation titers for anaplasmosis. Blood samples were collected for this purpose twice a week a minimum of 14 days before and 60 days after treatment. The relapse period is expressed as days following treatment required for the development of a 1% or greater A. marginale parasitemia. Except for 3 calves in Table 3A, infectivity trials were conducted on all animals, not showing an A. marginale relapse, by an I.V. injection of 200 ml of whole blood from the treated animal into a susceptible splenectomized calf. With the exception of Group I and II the time at which infectivity trials were conducted was never less than 60 days and on an average was 87 days. Groups I and II were tested for infectivity 5 to 20 days after treatment. In some instances blood from 2 and 3 calves was pooled and injected into 1 splenectomized calf.

Results:

The results following the use of 356 C 61 alone are presented in Table 1. Treatment with 5 mg/kg of 356 C 61 for 5 consecutive days failed to eliminate the A. marginale infection. The use of 5 mg/kg 356 C 61 injected 3 and 4 times at 2, 3-4, 7 and 14 day intervals was equally unsuccessful. Treatment for 10 consecutive days with 5 mg/kg 356 C 61 was apparently successful in removing evidence of A. marginale based on infectivity tests, but resulted in fatal toxicosis in every animal.

The results of combined therapy with 356 C 61 and oxytetracycline are presented in Table 2. Group IV, using only 2 mg/kg 356 C 61, in combination with a constant of 11 mg/kg oxytetracycline, failed to prevent an A. marginale relapse. Groups V and VII, using 5 or 10 mg/kg 356 C 61 in combination with oxytetracycline for 3 times at either a 24 or 48 hour interval eliminated evidence of infection based on the absence of a relapse and negative infectivity trials. Group VI given similar drug levels 3 times at 72 hour intervals was less effective, and 1 of the 3 treated calves developed an A. marginale relapse 39 days after the last treatment. Group VIII given only 2 injections of similar drug levels at either 24 or 48 hour intervals failed in every case to prevent relapse infection.

The results of 1 injection of 4A65 alone and in combination with 356 C 61 and oxytetracycline are presented in Table 3. All calves receiving only 1 injection, showed a relapsing infection an average of  $42 \pm 7$  days after treatment.

The results of 3 injections at 24 hour intervals of 4A65 alone and in combination with 356 C 61 are presented in Table 3A. In every instance evidence is present to indicate an elimination of A. marginale. Infectivity trials are complete in 4 of 7 calves, and the 3 remaining animals have shown no sign of relapse for over 60 days.

Discussion and Conclusions:

A combination of oxytetracycline and 356 C 61 appears more effective than either given alone. It would appear from these results that this action represents more than just an additive effect, and that there may be a synergistic effect in this instance. Using the drug levels reported, 4A65 appears equally as competent to eliminate infection when given alone or in combination with 356 C 61. This drug when given I.V. was responsible for excessive salivation, lacrimation and labored breathing. These side effects appear less severe when the drug is administered I.M. or S.C., and the efficacy was not altered.

Relapses when they occur usually do so between 30-45 days. The longest relapse period observed to date has been 62 days. The occurrence of a diagnostic relapse negates the need for infectivity trials. Our experience has been that treated calves, failing to show a relapse within a 62 day period are consistently free of infection as determined by infectivity trials.

These studies suggest that marked improvements are possible in treatment techniques, however much needs to be done before these drugs can be freely used and dispensed. Toxic side effects, tissue residues, as well as trials in intact animals are indicated. Hopefully such trials will result in the evolution of more effective and efficient drugs for the elimination of infection.

Summary:

Two new drugs,  $\alpha$ -dithiosemicarbazone (356 C 61) and 3,3'-Bis(2-imidazolin-2-yl) - carbanilide dihydrochloride (4A65) have been successfully used to treat splenectomized calves with anaplasmosis. Carrier infections were eliminated with 5 or 10 mg/kg 356 C 61 and 11 mg/kg oxytetracycline when given 3 times at either a 24 or 48 hour interval. In addition 5 mg/kg 356 C 61 plus 2 mg/kg

4A65 given 3 times at 24 hour intervals was effective in eliminating A. marginale infections. Levels of 4 and 6 mg/kg of 4A65 given 3 times at 24 hour intervals has proven successful in eliminating A. marginale infection.

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**Table 1**  
**Use of 356 C 61 to Eliminate Carrier Status in Splenectomized Calves**

	No. of Animals	Dose Rate mg/kg	Route of Inoculation	Number of Inoculations	Interval of Inoculations	Results of Treatment	Infectivity Trials
Group I	3	5	I.V.	5	24 hrs	Failed	Positive
Group II	3	5	I.V.	10	24 hrs	Successful	Negative*
Group III	8	5	I.M.	3 & 4	2, 3-4, 7 and 14 days	Failed Relapse: 40 ± 11 da	N.T.

\* All calves treated with 356 C 61 - 10 times at the 5 mg/kg level died, apparently due to drug toxicity.

N.T. - No Test

I.V. - Intravenous

I.M. - Intramuscular

**Table 2**

Use of 356 C 61 in Combination With Oxytetracycline to Eliminate Anaplasma Carrier Status of Splenectomized Calves

	No. of Animals	Dose Rate mg/kg		Route of Inoc.	No. of Inoc.	Interval of Inoc.	Results of Treatment	Relapse Time Days	Infectivity Trials
		356 C 61	Oxytet.						
Group IV	3	2	11	I.V.	3	24 or 48 hrs	3/3 Failed	31.7 ± 14.1	N.T.
Group V	11	5	11	I.V.	3	24 or 48 hrs	11/11 Successful	-----	11/11 Negative
Group VI	3	5	11	I.V.	3	72 hrs	2/3 Successful	39.0	2/3 Negative
Group VII	8	10	11	I.V.	3	24 or 48 hrs	8/8 Successful	-----	8/8 Negative
Group VIII	8	5 or 10	11	I.V.	2	24 or 48 hrs	8/8 Failed	41.9 ± 9.6	N.T.

I.V. - Intravenous

N.T. - No Test

Table 3

Use of 4A65 Alone and in Combination With 356 C 61 to Eliminate  
The Anaplasma Carrier Status of Splenectomized Calves

Group IX

Calf Number	4A65			356 C 61			Result of Treatment	Relapse Time In Days	Infectivity Trial
	mg/kg	Route	No. of Inoc.	mg/kg	Route	No. of Inoc.			
405*	4	I.M.	1	15	I.V.	1	Failed	42	N.T.
135	6	I.M.	1	15	I.V.	1	Failed	54	N.T.
219	6	I.M.	1	15	I.V.	1	Failed	43	N.T.
424	15	S.C.	1	--	----	-	Failed	38	N.T.
431	15	S.C.	1	--	----	-	Failed	35	N.T.
								(42 ± 7) Days	

\* Treated simultaneously with 44 mg/kg oxytetracycline I.V.

I.M. - Intramuscular

I.V. - Intravenously

N.T. - No Test

S.C. - Subcutaneously

Table 3A

Multiple Injections of 4A65 Alone and in Combination With 356 C 61 to Eliminate The Anaplasma Carrier Status of Splenectomized Calves

Group X

Calf Number	4A65				356 C 61				Relapse Time In Days	Infect. Trial
	mg/kg	Route	No. of Inoc.	Interval	mg/kg	Route	No. of Inoc.	Interval		
217	2	I.M.	3	24 hrs	5	I.V.	3	24 hrs	>120	Neg.
248	2	I.M.	3	24 hrs	5	I.V.	3	24 hrs	>120	Neg.
450	5	I.M.	3	24 hrs	2	I.M.	3	24 hrs	>120	Neg.
245	4	I.M.	3	24 hrs	---	----	----	-----	>120	Neg.
405	5	S.C.	3	48 hrs	---	----	----	-----	>120	Neg.
413	5	S.C.	3	48 hrs	---	----	----	-----	>90	Neg.
265	6	I.M.	3	24 hrs	---	----	----	-----	>120	Neg.

I.M. - Intramuscular

I.V. - Intravenous

N.T. - No Test

Addendum To:

Promising Therapeutic Agents for the Elimination  
of Anaplasma marginale in the Carrier Animal

A continuation of the studies reported in the paper "Promising Therapeutic ...." has produced less than favorable results in some instances. In Table 3A Calf 450 (treated with both 4A65 and 356 C 61) was proven free of infection by infectivity trials consisting of the inoculation of 200 ml of its blood into a susceptible splenectomized calf. Similar trials were conducted on Calves 405, 413, and 465, all receiving only 4A65 given in 5 and 6 mg/kg levels 3 times at 24 to 48 hour intervals. In all 3 instances these calves were proven free of infection.

Additional trials using 4A65 alone are reported in Table 4. Splenectomized Calf 272 was treated 3 times at 24 hour intervals, with 2 mg/kg injected s/c. Calf 439 was treated 3 times at 24 hour intervals with 3 mg/kg injected s/c. Calves 458, 457, and 431 were each treated 3 times at 24 hour intervals with 5 mg/kg injected s/c. Calf 437 received the same treatment but at a 48 hour interval. Intact Calf 401 was treated 3 times at 24 hour intervals with 5 mg/kg, s/c. This calf was checked for residual infectivity by splenectomy 90 days after treatment.

Calves 404, 429, and 436 were each treated 2 times at a 14 day interval with 5 mg/kg injected s/c.

Calf 272, receiving 2 mg/kg, relapsed in 67 days. Calf 458 relapsed in 53 days. Calves 457 and 431 both died, the cause of death being unknown, but drug toxicosis was suspected. Calf 404, receiving 2 treatments at a 14 day interval, relapsed, 34 days after the last treatment.

Calves 439 (3 mg/kg - 3 times at 24 hours interval), Calf 437 (5 mg/kg - 3 times at 48 hours interval), Calf 401 (5 mg/kg - 3 times at 48 hours interval),

Calf 436 (5 mg/kg - 3 times at 24 hours interval) have been shown free of infection. Calf 429 will be tested soon for infectivity, but at present is still under observation for possible relapse.

Adult intact Cows 409, 417, and 419, all carriers of anaplasmosis were treated with 4A65 to determine the influence of each treatment on the carrier status. All cattle were checked for carrier status by the CF test and by calf inoculation into splenectomized calves. All 3 cows were very old, and in poor flesh, reflecting the effects of poor pasture conditions and insufficient supplementary feed.

On day 0 Cow 409 was treated with 5 mg/kg, 4A65, injected subcutaneously. The CF titer was 1:80 at the time of treatment. On days 2 and 4 the treatment was repeated. The cows weight was 610 pounds. On day 39 the cow died and was necropsied. Prior to death marked sub-mandibular and facial edema was noted. There was marked lacrimation and swelling about the eyes. A BUN conducted 17 days before death was 43 mg%. Necropsy findings were clouded by advanced autolysis, but there was edema throughout, excessive fluids in the peritoneal and thoracic cavities, and a degree of nephrosis. It is probable that drug toxicosis played a role in the death of this animal, however, it is not unlikely that the advanced age, and poor condition of the animal contributed to the fatal reaction.

Cow 419S was treated on day 0 and again on day 14 with 5 mg/kg 4A65 injected s/c. On day 16 Tramisol was administered for the control of internal parasitic infestation. The BUN on day 21 was 72, on day 41 it was 130. Death occurred on day 41. Prior to death Cow 419S showed marked sub-mandibular and facial edema. Severe lacrimation, and marked keratitis in both eyes was present to the extent that the cow was almost blind, and it had apparently been in this situation for quite some time. Necropsy showed severe nephrosis, peritoneal and thoracic exudates. Again drug

(24)

toxicosis is suspected, but secondary factors may have contributed to death.

Cow 417 was treated on day 0, 7, and 14 with 5 mg/kg 4A65, injected s/c. The BUN on day 41 was 34, and on day 48 was 26. Cow 417 showed some facial edema, but not as marked as seen in Cows 409 and 419. Cow 417 was checked for infectivity on day 41. As of day 65, other than general weakness and poor condition it shows no signs of drug intoxication, or evidence of Anaplasma relapse.

Table 4

**Treatment With 4A65 for The Elimination of Carrier Infection  
(Subcutaneous Injections)**

Calf Number	mg/kg	No. of Treatments	Interval	Relapse Time (1) (Days)	Infectivity Trials
272	2	3	24 hrs	67	----
439	3	3	24 hrs	>158	Neg.
401(2)	5	3	24 hrs	>120	Neg.
458	5	3	24 hrs	53	----
457	5	3	24 hrs	Died*	----
431	5	3	24 hrs	Died*	----
437	5	3	48 hrs	>90	Neg.
404	5	2	14 da	34	N.T.
429	5	2	14 da	>60	N.T.
436	5	3	14 da	>102	Neg.

N.T. - No Test.

\*Died 6 and 15 days after last treatment - Suggestive of Drug Toxicosis.

(1) - After last treatment.

(2) - Intact calf (splenectomized 90 days after treatment, no sign of relapse).

Repeated Administration of Therapeutic Agents for  
the Prevention and Elimination of Anaplasma Infection

A proven method for eliminating or preventing anaplasmosis is the continuous low level feeding of tetracyclines daily for 60 to 90 days. Daily injections of tetracyclines, at the rate of 5 mg/lbs, for 10 to 14 days is also reported to be effective in eliminating infection. These procedures while useful, are not 100% effective, and require a prolonged course of treatment which is both time consuming and expensive. This treatment is the best now available and is being used in some anaplasmosis control programs. The reactor cows are removed from the herd, treated and then returned to the herd.

Using splenectomized calves studies have been made with several variations, to better evaluate not only the tetracyclines but also 4A65 when administered over an extended period of time. Splenectomized calves have been used to more severely test the value of the various therapeutic agents. The anaplasmosis response in splenectomized calves closely resembles the disease in adult cattle, and such calves are more consistently susceptible than adult cattle. It is probable that the splenectomized calf is unable to elicit a marked immune response to infection, and that which does occur is inadequate. The response of the Anaplasma organism to specific drugs can be measured in these animals with a minimum of influence by the animals normal protective mechanisms. The general assumption is that if treatment is effective in this host it will be even more effective in intact animals, probably at a lower dose range.

Both preventive therapeutic measures and treatment procedures to remove the carrier infection have been tried.

Preventive Procedures:

The addition of tetracyclines to salt has been suggested as a possible preventive measure. It is questionable that sufficient tetracyclines would be ingested by this means. If tetracyclines were used at the rate of .5 mg/lbs, 5 grams daily would be required for a 1,000 pound animal. Used at the rate of 0.1 mg/lbs, only 1 gram daily would be required for a 1,000 pound animal. Considering that adult cattle will consume approximately 10 grams of salt daily (Morrison's Feeds & Feeding) it would be feasible to obtain an intake of 1 to 2 grams of tetracycline daily. At current prices, 1 gram of Aureomycin costs approximately \$0.125. A therapeutic and preventive dose at 0.5 mg/lbs would cost the producer \$0.62 a day or \$37.50 for a 60 day feeding trial.

Prevention Experiment #1:

Three splenectomized calves were used in this experiment, Calf 495 (figure 1), was fed chlortetracycline at the rate of 0.1 mg/lbs body weight daily beginning 14 days before Anaplasma exposure, which consisted of the injection, s/c, of 1 ml whole blood from Calf 460, a splenectomized carrier. A similar exposure was given weekly to simulate the continuous exposure expected under natural conditions. Treatment and exposure continued until day 44, when the experiment was terminated due to the death of 495 from acute anaplasmosis.

Calf 488 (figure 2), was fed chlortetracycline at the rate of 0.5 mg/lbs body weight daily beginning 14 days before anaplasmosis exposure. This was accomplished by adding Aureomycin crumbles (Cyanamid) to the daily grain ration in amounts calculated to give the desired dose rate. Calf 488 was challenged on day 14 and every 7 days thereafter, by the s/c injection of 1 ml blood from Calf 460. On day 35 an increase in CF titer was detected, this was followed by a marked increase in an Anaplasma parasitemia, and a drop in PCV. After dropping to 15% the PCV showed signs of returning to normal. Observations of this calf are reported up to 84 days, and are continuing. It is planned to continue the present course of treatment, and periodic exposure for at least another 80 days.

Calf 404 (figure 3), served as an untreated control, inoculated s/c with 1 ml blood from Calf 460. First diagnostic evidence (1% Anaplasma parasitemia, or a 4+ CF response) was detected on day 21. This was followed by a 58% parasitemia, a high CF titer of 1:640, and a drop in PCV to 7%. Following a low PCV of 7% the animal recovered.

Discussion and Conclusions:

Chlortetracycline, at the rate of 0.1 mg/lbs, failed to prevent acute anaplasmosis. It may have retarded slightly the onset of disease when

compared with the untreated control. Chlortetracycline at the rate of 0.5 mg/lbs while not preventing anaplasmosis did modify the severity of infection when compared with the untreated control. Calf 483 will be followed for an additional 80 days to determine whether or not continuous feeding will inhibit characteristic relapses, and possibly eliminate the infection. Franklin and others have observed that 0.5 mg/lbs fed daily for 60 to 90 days will in some instances eliminate the carrier state. These results suggest that, in splenectomized calves, 0.1 and 0.5 mg/lbs chlortetracycline is insufficient to prevent clinical anaplasmosis.

Figure 1 Calf 495

Prevention - Low level (0.1 mg/lbs.) Aureomycin - daily - per os.

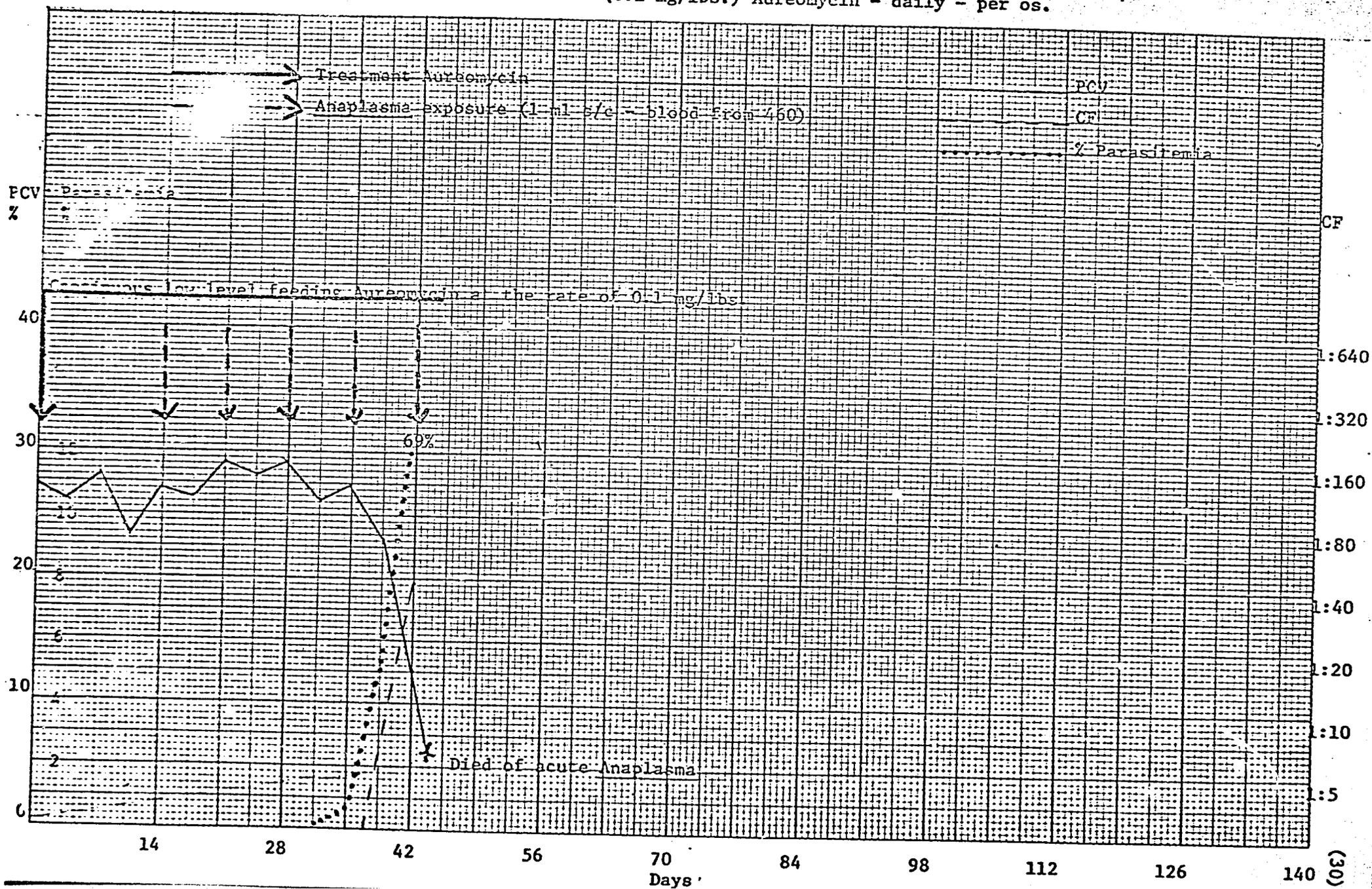


Figure 2 Calf 488

Prevention - Continuous low level Aureomycin

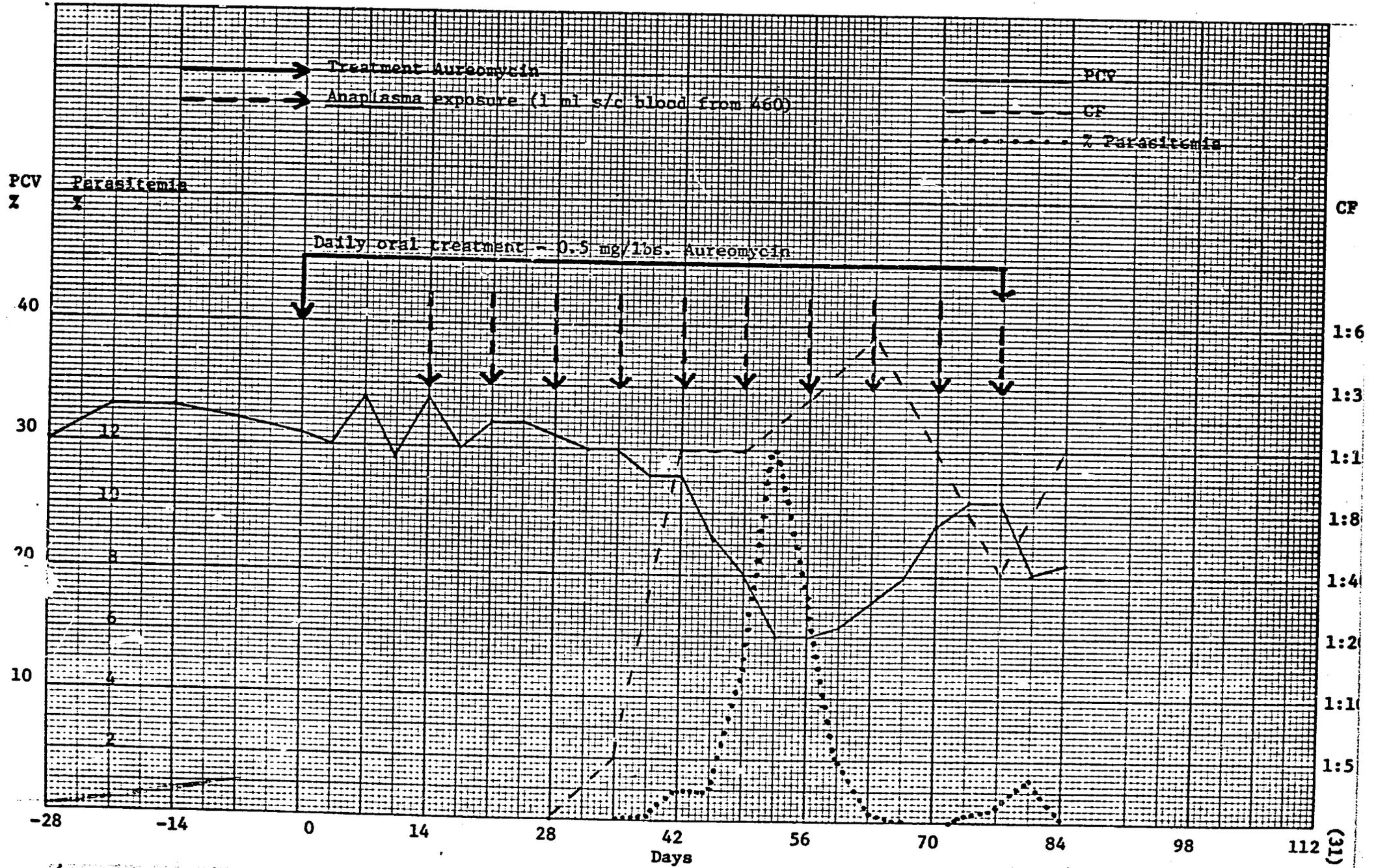
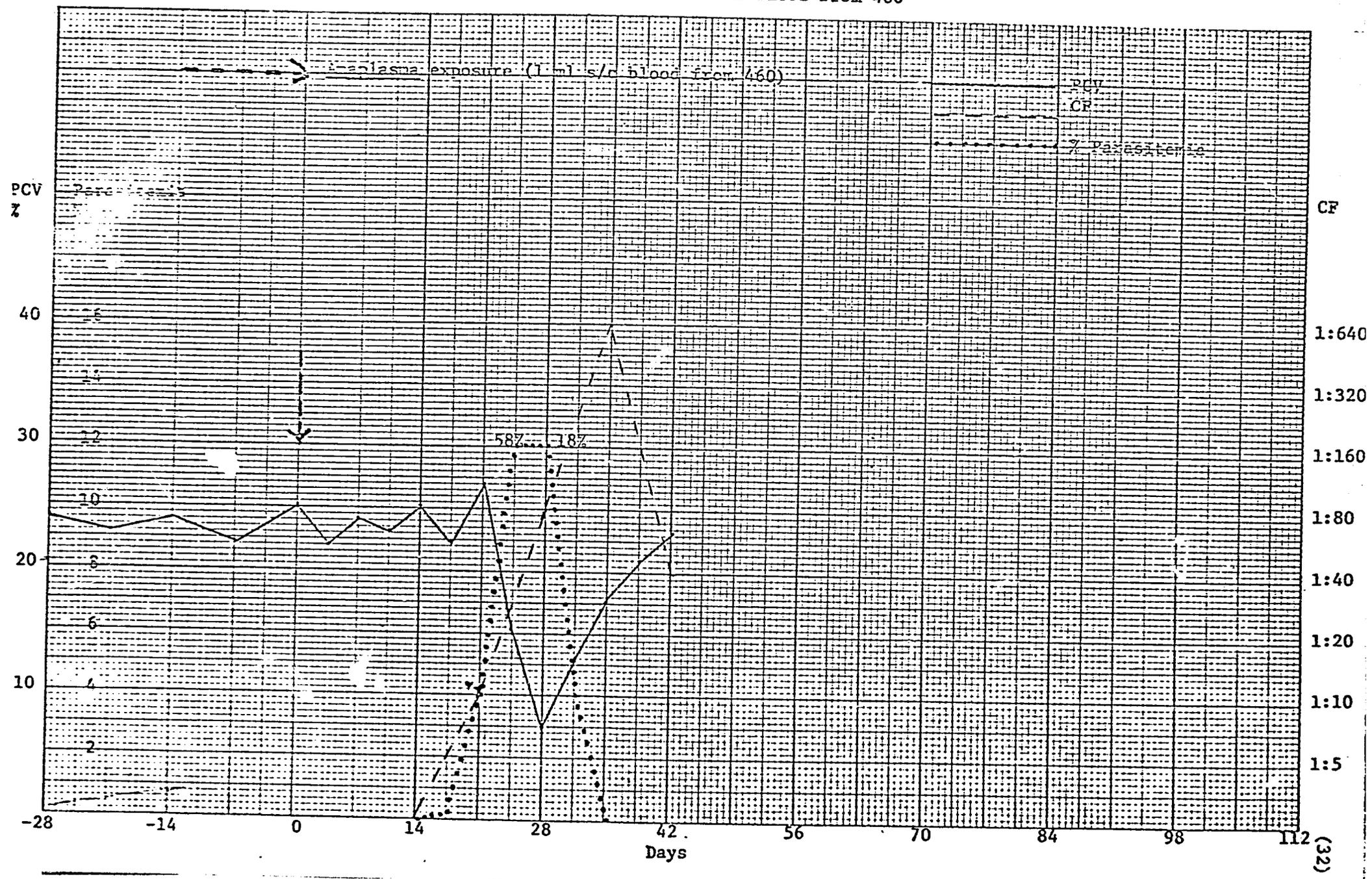


Figure 3 Calf 404

Control - Inoculated - 1 ml blood from 460



Prevention Experiment #2:

In view of the apparent anaplasmaicidal effects of 4A65 under some conditions, it was decided to use this agent as a prophylactic drug in the presence of weekly Anaplasma exposure.

Calf 434 (figure 4), was exposed by the s/c injection of a 2 ml inoculum consisting of 50% whole blood from an acute case of anaplasmosis which had been frozen with equal parts 6% DMSO in physiologic saline in August, 1969. The injection was made immediately after thawing. Drug treatment consisted of the s/c administration of 5 mg/kg 4A65, given on days 0, 28, 56, and 84. First evidence of anaplasmosis occurred on day 119, 35 days after the last treatment, and 49 days after the last inoculation of infectious material.

Calf 433 (figure 5), was exposed to anaplasmosis in the same manner as described for 434. Treatment was similarly administered on days 0, 28, and 56. No evidence of anaplasmosis was seen in Calf 433 at day 140. On day 160 Calf 433 was challenged by the s/c injection of 1 ml whole blood freshly drawn from 460. Evidence of infection developed 19 days after challenge, and the animal proved to be fully susceptible.

Calf 439 (figure 6), was an untreated control, exposed to identical infecting inoculums as described for Calf 434. The first evidence of infection occurred on day 35. Typical elevated CF titers, and low PCV values associated with an Anaplasma parasitemia characterized the control response, and demonstrated the viability of the inoculating organism. On day 105 Calf 439 was given a series of 3 injections at 24 hour intervals consisting of 5 mg/kg 4A65. A marked increase in PCV's was noted, and on later tests this calf was found to be negative for Anaplasma.

Conclusion:

Treatment with 5 mg/kg 4A65 at 4 week intervals was partially

successful in preventing anaplasmosis. The long incubation period (35 days) observed with the control does suggest that the challenge inoculum was of low titer. Evidence of infection was, however, prevented in 1 animal and retarded in a second. This approach may have merit in a situation where premunition is desirable, but the severe effects of infection need to be minimized. There is a strong possibility that continuous treatment as described might entirely block infection in the intact calf if exposures were moderate in nature. This is suggested by the prevention in splenectomized Calf 433.

Figure 4 Calf 434

Prevention - Periodic treatment - Weekly exposure

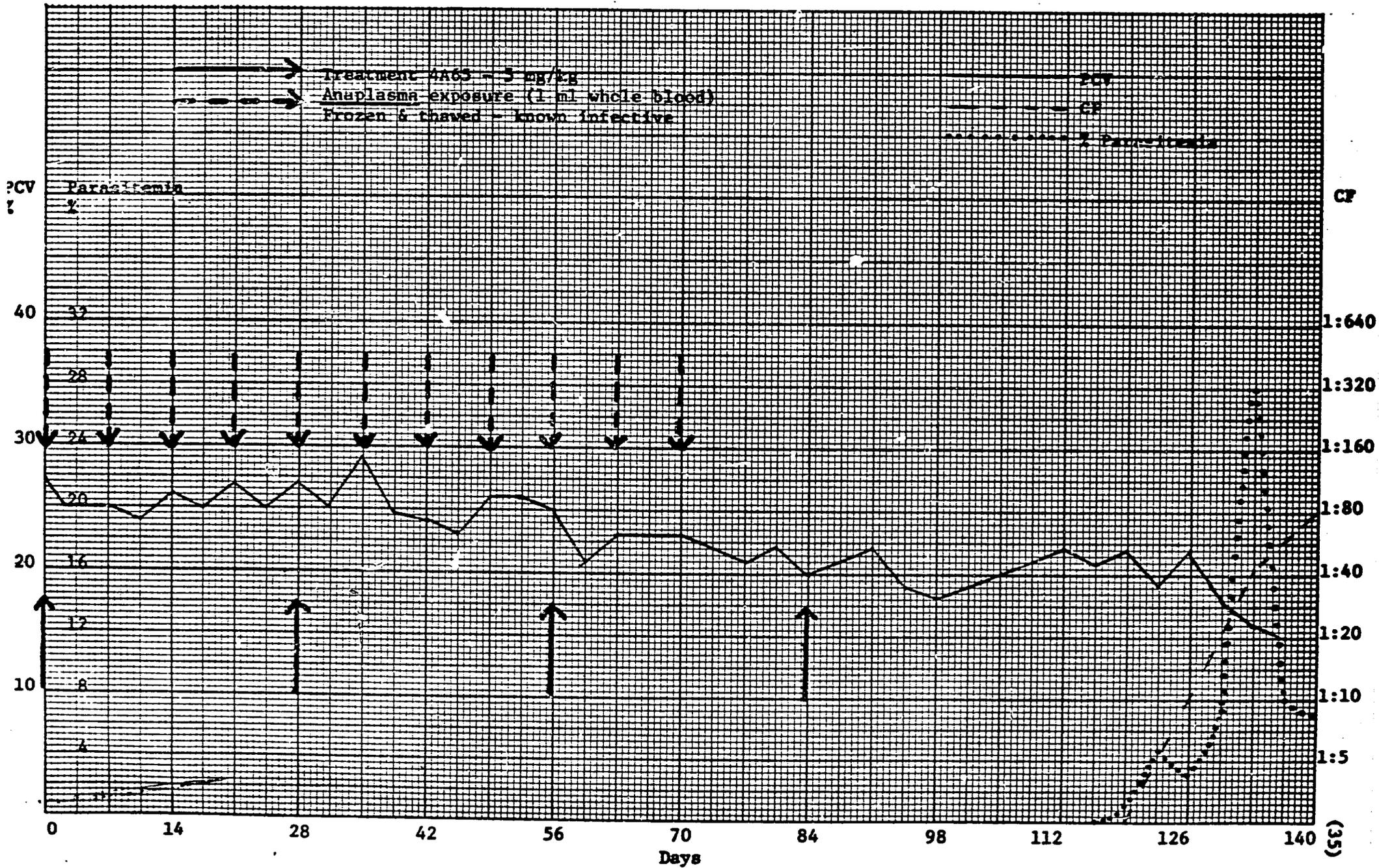


Figure 5 Calf 433

Prevention - 5 mg/kg - s/c - 4A65 given at 4 week intervals

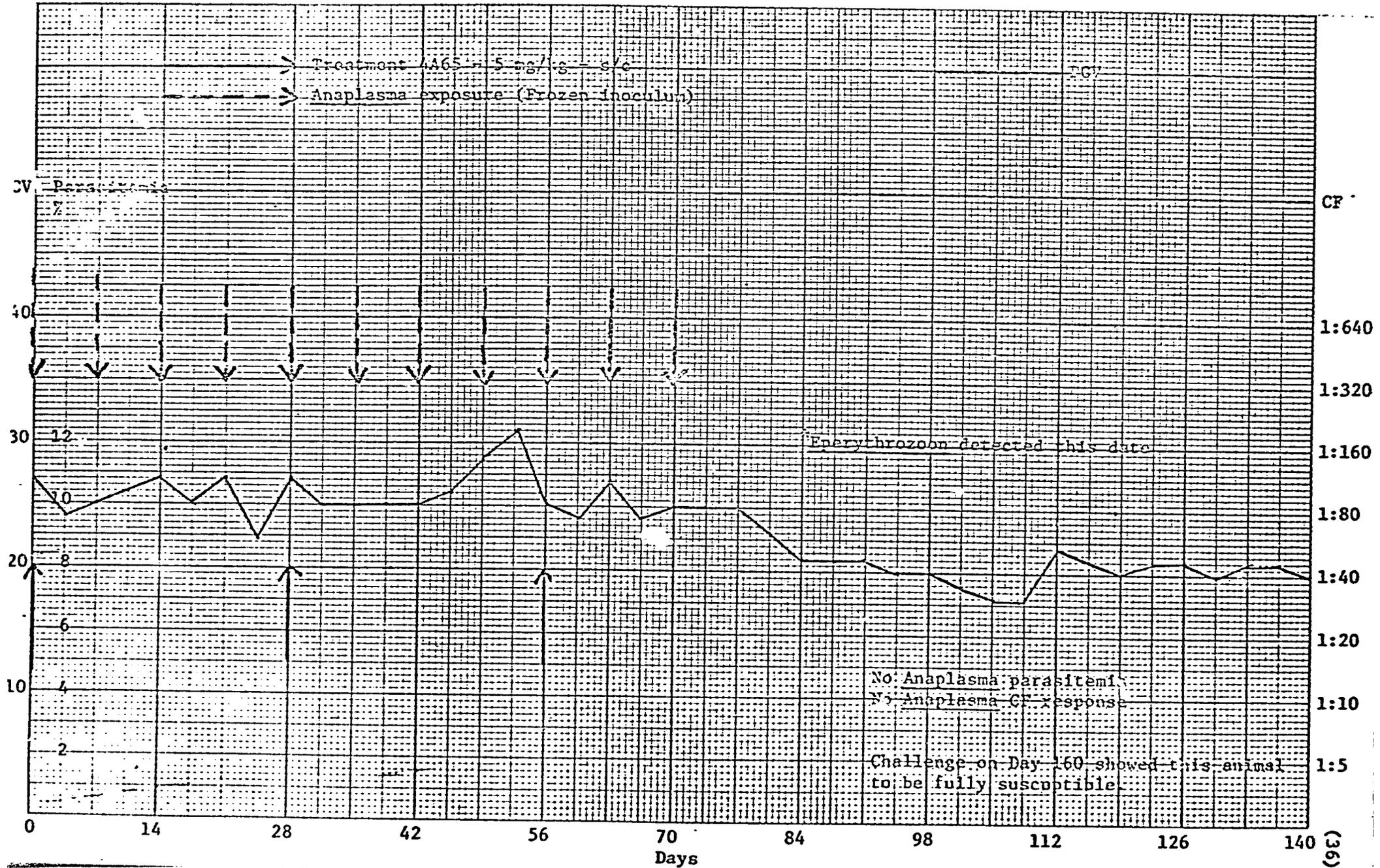
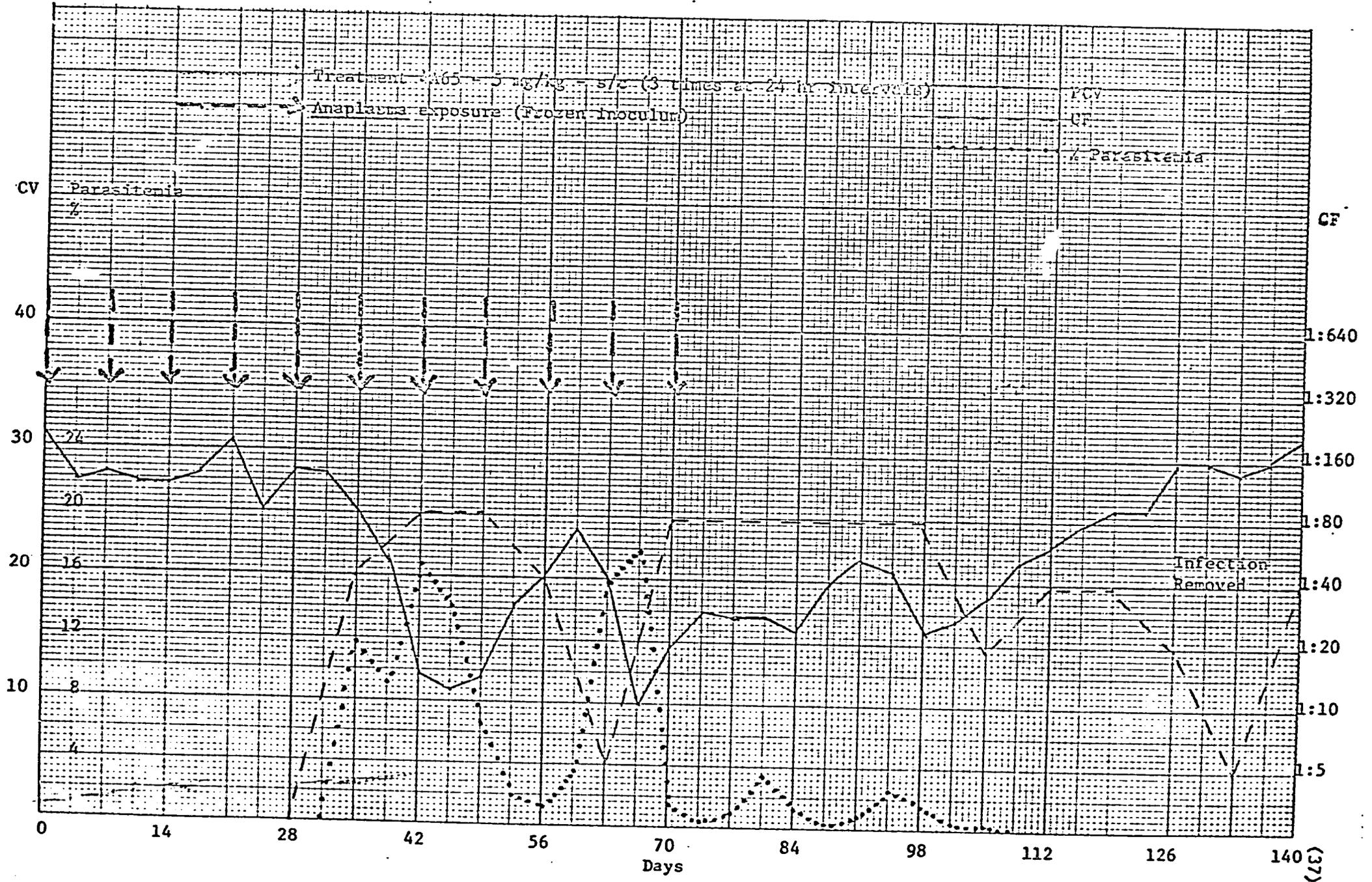


Figure 6 Calf 439

Control



Prevention Experiment #3:

The relatively long incubation time for the control Calf 439 in Experiment #2 suggests that a low level challenge was being used in this instance. This experiment was essentially the same as #2, except that the infecting inoculum was freshly drawn blood from a splenectomized carrier calf.

Calf 461 and 462 (figures 7 and 8), were treated the same in every respect. The infecting inoculum was given weekly beginning on day 28, and consisted of 1 ml blood, freshly collected from a splenectomized carrier calf (460), and injected s/c. The first 4 infections (days 0, 7, 14 and 21) were composed of material that had been frozen (the same as used for Calf 434). Treatment consisting of injecting s/c 5 mg/kg 4A65 every 4 weeks or 6 times for the 140 day period.

Both Calves 461 and 462 developed evidence of anaplasmosis, after a long incubation period. Calf 461 showed signs of infection on day 84. This response was mild, being characterized by a high CF of 1:640, a high parasitemia of 3% and a low PCV of 15%, which was 62% of normal. This is considered a mild response to infection induced in splenectomized calves by artificial needle challenge. Calf 462 developed first signs of infection on day 63. The resulting infection was mild in that the high parasitemia was only 3%, the low PCV was 20% (or 55% of normal) and a high CF titer of 1:100 occurred. The anemia did not persist, but the animal rapidly recovered.

The series of weekly exposure and treatment every 4 weeks is continuing. The untreated control for this experiment was Calf 404 (figure 3). Calf 404 developed evidence of infection on day 21, characterized by a parasitemia of 58% and a high CF titer of 1:640, and a drop to 7% PCV (29% of normal).

Figure 7 Calf 461

Prevention - By continuous treatment

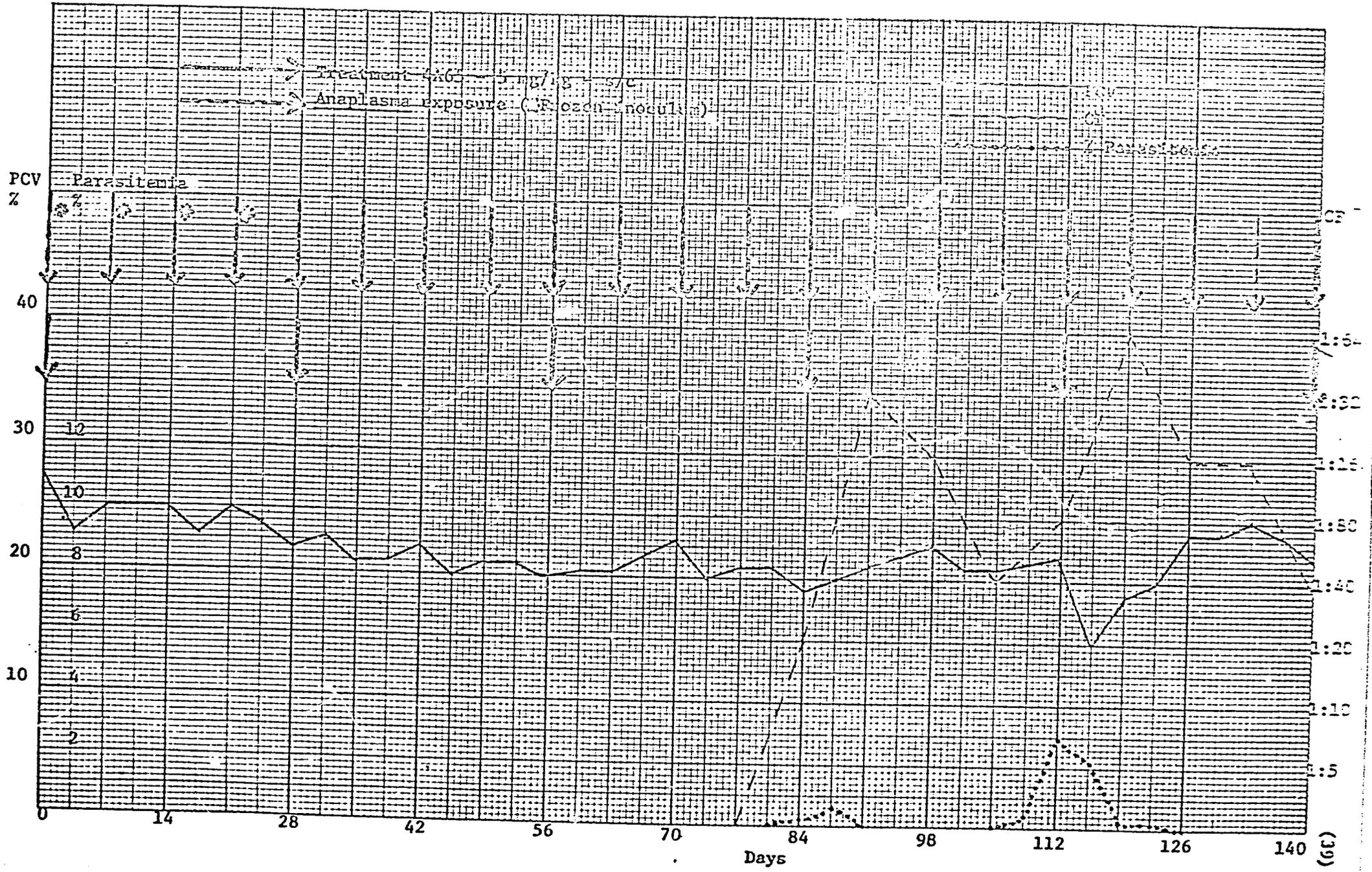
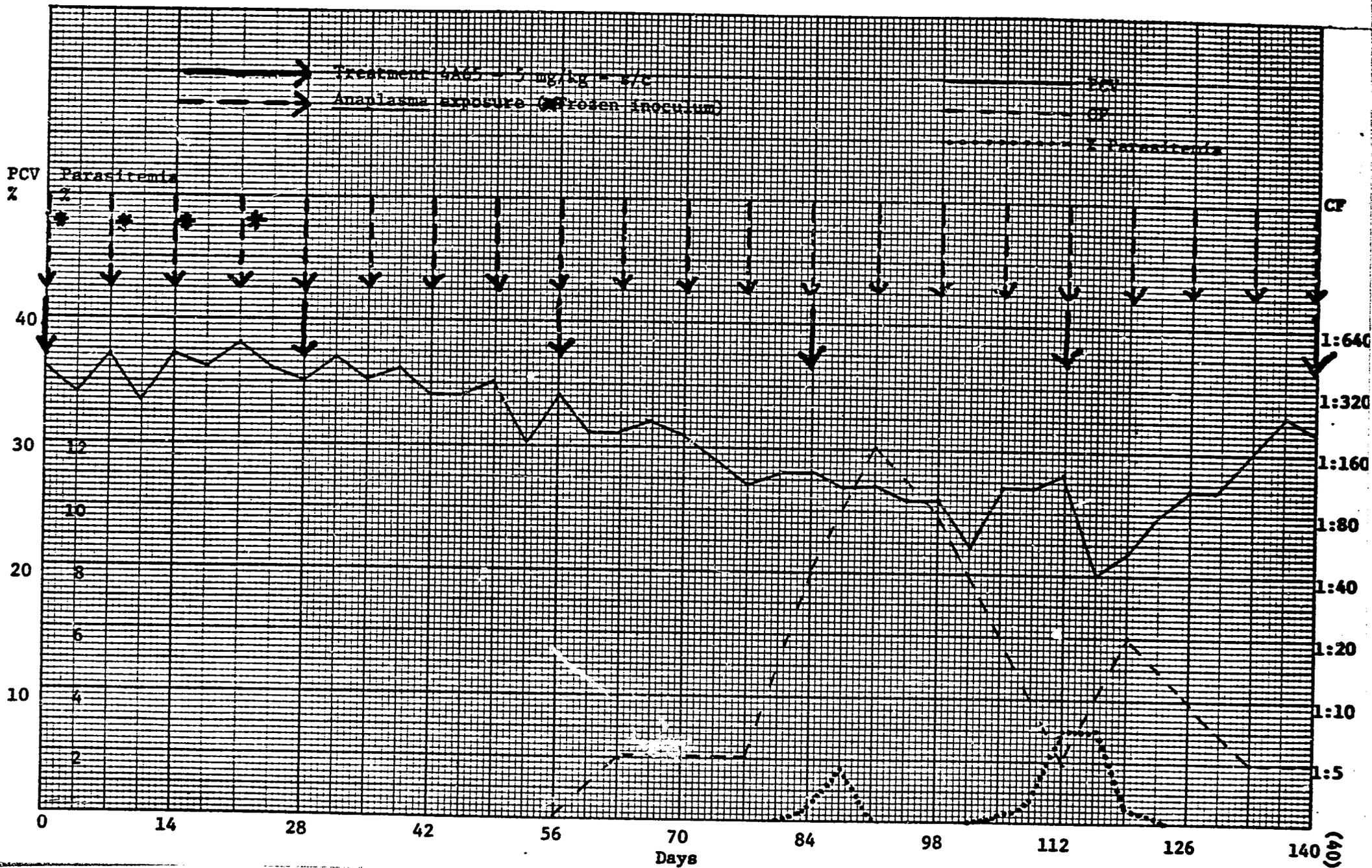


Figure 8 Calf 462

Prevention by continuous treatment



Prevention Experiment #4:

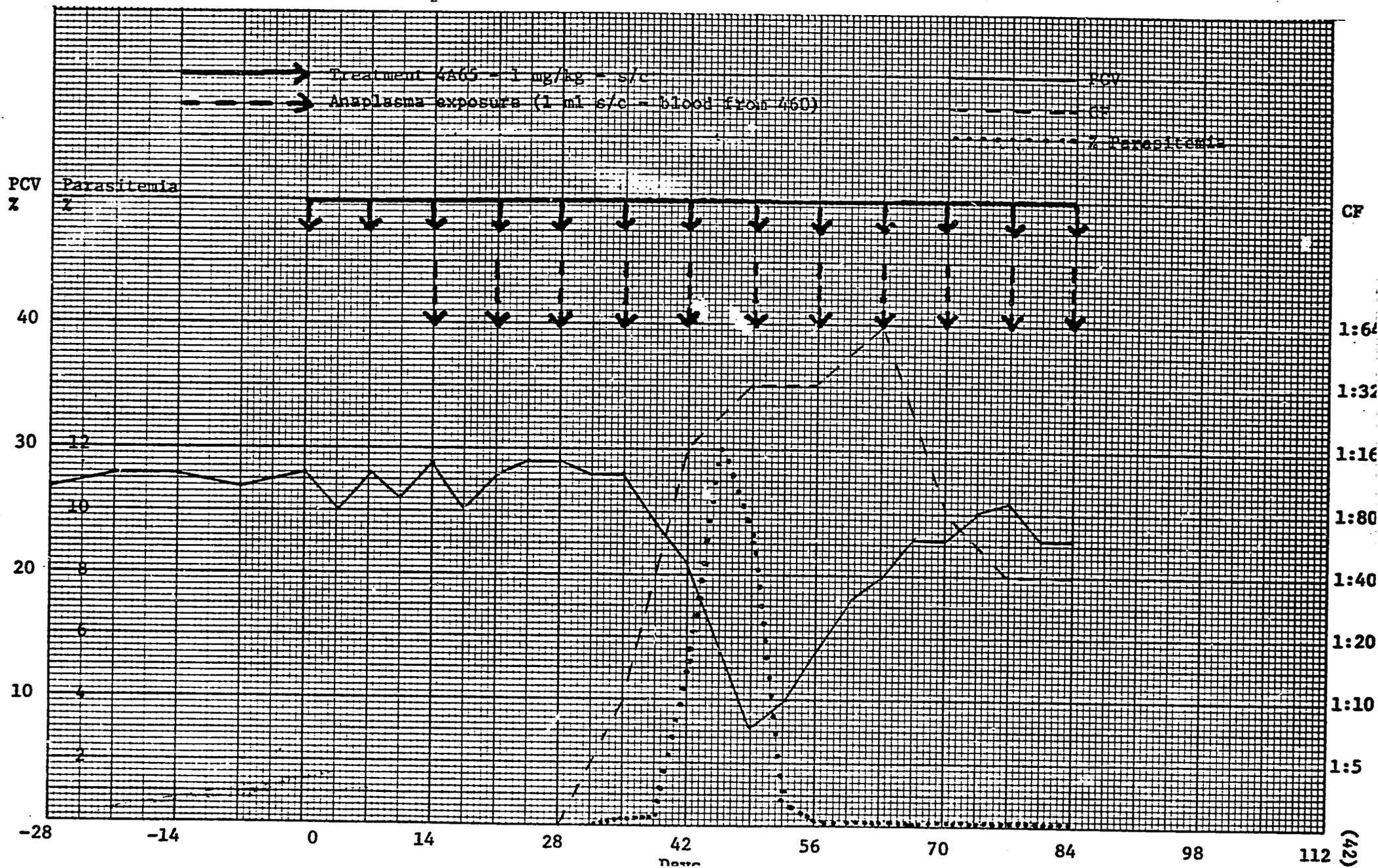
This experiment employs a minor variation of experiments 2 and 3, in that the 4A65 will be given weekly not every 4 weeks.

Beginning on day 0, Calf 490 (figure 9), was treated weekly with 1 mg/kg, 4A65 administered s/c. Calf 490 was infected at weekly intervals beginning on day 14 by the injection of 1 ml freshly drawn blood from Calf 460. The first evidence of anaplasmosis was observed 21 days after the initial anaplasmosis exposure. The reaction was characterized by a drop in PCV to 8%, or 32% of normal, a marked serologic response to the CF test associated with a high Anaplasma parasitemia.

No evidence of drug effect was detectable when the reaction is compared to Calf 404, the untreated control. This animal was given approximately the same amount of 4A65, as proved effective in Calves 461 and 462, but the treatments were given at weekly not 4 week intervals. It might be possible to surmise that 4A65 is more effective when high blood levels are reached, rather than lower sustained blood levels maintained over a period of time.

Figure 9 Calf 490

Prevention - Weekly injections of 4A65



Treatment Experiment #5:

This study involves the use of chlortetracycline fed daily at varying dose levels, and for different times, to splenectomized Anaplasma carrier calves to measure the relative effect of such treatment on parasitemia, PCV, and CF titer, when compared to non-treated controls.

Calf 434 (figure 10), was fed chlortetracycline at a rate of 0.1 mg/lbs for 28 days. A severe Anaplasma relapse at this time suggested that this dose level was insufficient to control the cyclical manifestation of Anaplasma. For this reason the treatment level was increased to 0.5 mg/lbs for an additional 102 days. During this time mild relapses were observed at about 45 days, and again at day 105. A gradual regression of CF titers was noted along with a marked increase in PCV which stabilized at about day 56. A marked relapse occurred in 434 about 24 days after the discontinuance of chlortetracycline therapy, which was characterized by a PCV of 18%, a 4% parasitemia, and an increase in CF titer to 1:80.

Calf 279, (figure 11), was fed chlortetracycline at the rate of 0.5 mg/lbs, daily for 60 days. There was no parasitemia evident throughout the treatment period, and the CF titer declined from a titer of 1:80 to negative within 66 days. No significant fluxuations occurred in the PCV during the 140 day observation period other than a slight depression due to an Eperythrozoon infection which was controlled with neomycin. At day 126, infectivity trials, involving the transfer of 200 ml whole blood from 279 to a splenectomized calf, proved negative, indicating that in this instance 0.5 mg lbs was sufficient for the removal of infection.

Calf 450 (figure 12), was fed chlortetracycline at the rate of 1.0 mg/lbs for 70 days. Treatment was started at a time when the parasitemia was 3%, and was declining after a relapse infection. A parasitemia

did not recur during the treatment period, but a relapse infection was detected at 101 days or 31 days after treatment. The CF t/ter declined from a titer of 1:40 to 1:5 where it remained constant for the remaining period of observation. A favorable PCV response occurred during treatment, but a marked drop is anticipated to follow the recorded parasitemia on day 101.

Calf 424 (figure 13), was fed chlortetracycline at the rate of 5.0 mg/lbs daily for 60 days. This is the suggested level of treatment for carrier cows in the USDA eradication program, and is a level generally considered effective for the elimination of anaplasmosis. The parasitemia present at the onset of treatment disappeared during the treatment schedule. This was accompanied by a marked increase in PCV, and in a reduction in CF titer from 1:80 to 1:5. About 3 days after the discontinuance of treatment a marked parasitemia was seen. This developed a high of 6.5% before regressing.

Calf 191 (figure 14), a non-treated control shows marked re-cycling Anaplasma parasitemias with the accompanying drops in PCV and the maintenance of a fairly high level of CF activity. The cycle appears to repeat itself about every 30 days in a manner that is striking for its consistency.

#### Discussion and Conclusions:

When comparisons are made with the untreated control, it becomes obvious that chlortetracycline has specific activity in the treatment of Anaplasma. The 0.1 mg/lbs level was too low to effectively influence the growth of the Anaplasma organism in splenectomized calves. Growth inhibition was however detected at all other levels; 0.5, 1.0, and 5.0 mg/lbs. It is surprising that elimination of infection occurred in Calf 279 at a 0.5 mg level, whereas, 5.0 mg was unsuccessful in eliminating

the infection in 424. There are apparently other factors influencing the in vivo susceptibility of A. marginale than the dose level. Efforts to find a pattern, or history to explain this paradox have been unsuccessful. Every calf tested, including 279 had a history of previous treatment with 4A65. Chlortetracycline, had not been used in any of those animals tested. There is the possibility that prior exposure to 4A65 at a sub-lethal level may interfere with future drug activity, but there is only circumstantial evidence that this is the case. The obvious cycle patterns observed in infected calves also suggest the retention of infection in a form or at a time in its life cycle when it is resistant, or protected in some way from the effects of the therapeutic agent. This might explain the need for prolonged therapy, in order to eliminate infection. This cycling is not apparent in the intact animal, but probably does occur. The drug must be present when the organism becomes vulnerable, or else the carrier infection persists. This is partially substantiated by the disappearance of infection in Calf 279 treated 60 days at a 0.5 mg level, when treatment was started with no parasitemia evident. It is possible that treatment started at this time spanned a period of organism vulnerability. In Calf 424 treatment was started at the very end of an acute phase and to follow the assumption of cyclical vulnerability, it might be guessed that some dormant, or drug resistant forms had developed which persisted longer than the 60 day cycle. Treatment was stopped at about the same time that the parasitemia cycle recurred. These possibilities should be considered in establishing treatment programs for use in future eradication programs, and pose questions that must be answered by further research.

Failure to eliminate infection in splenectomized carrier calves confirms our earlier assumption that such animals would prove a more

difficult test for a therapeutic agent than would normally occur, and that an agent or compound effective in these experimental animals, would be effective against the intact animal and allow a considerable margin of safety.

Figure 10 Calf 434

Treatment - Continuous feed - Aureomycin - 0.1 mg/lbs. - Increased on Day 28 to 0.5 mg/lbs.

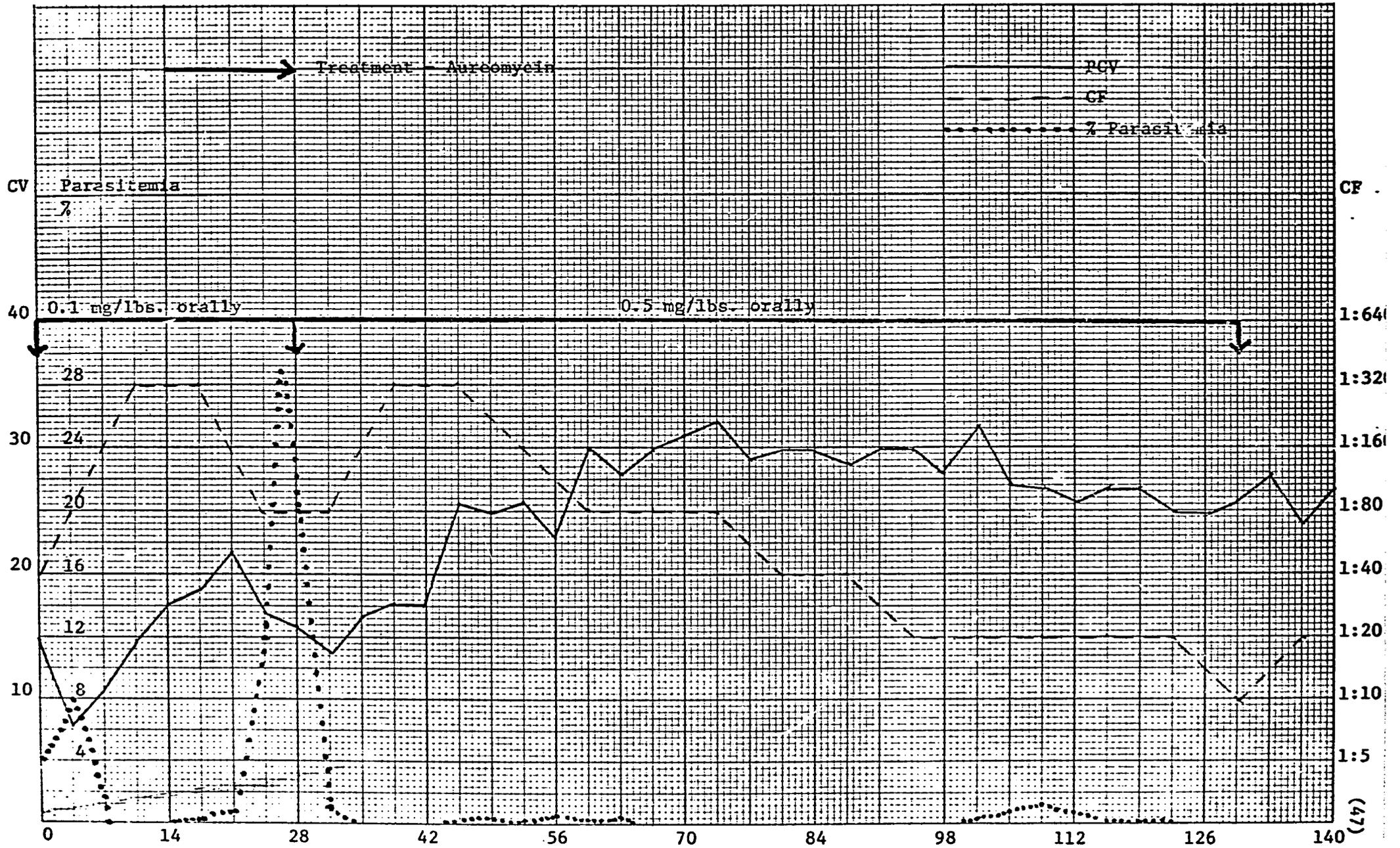


Figure 11 Calf 279

Treatment - Aureomycin - 0.5 mg/lbs. - daily - per os.

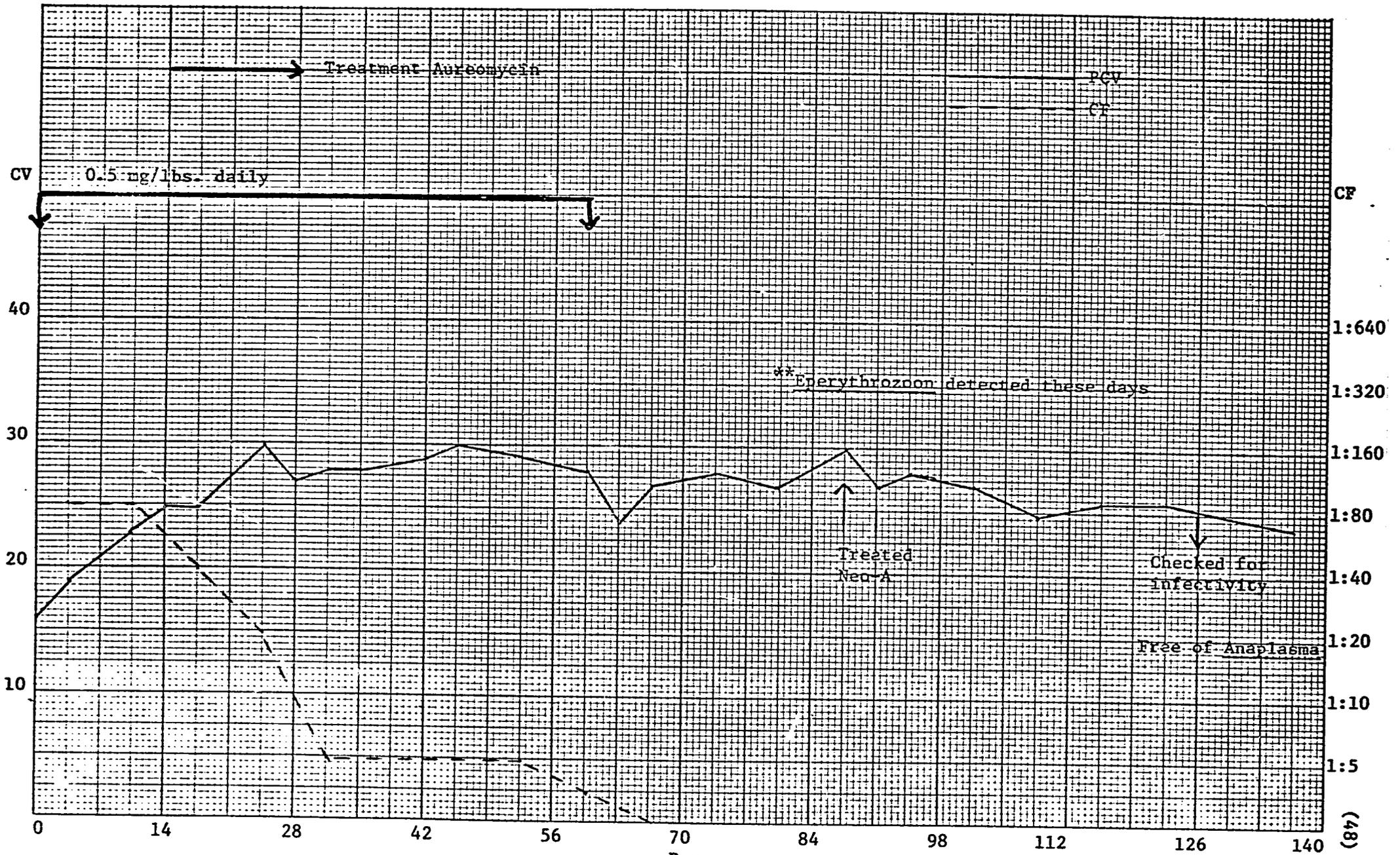


Figure 12 Calf 450

Treatment - Aureomycin - 1 mg/lbs. - daily - per os.

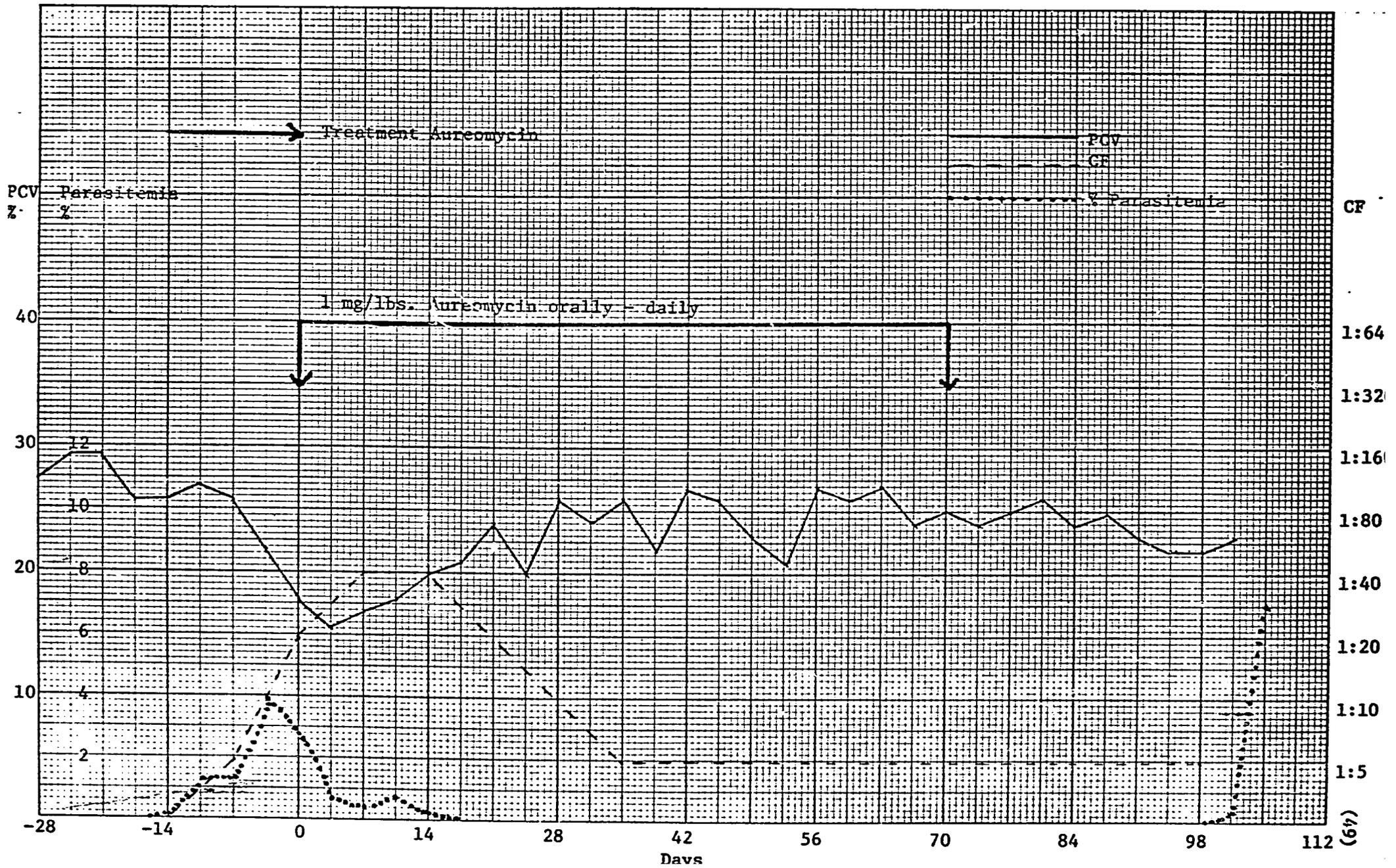


Figure 13 Calf 424

Treatment - Aureomycin - 5 mg/lbs. - orally for 60 days

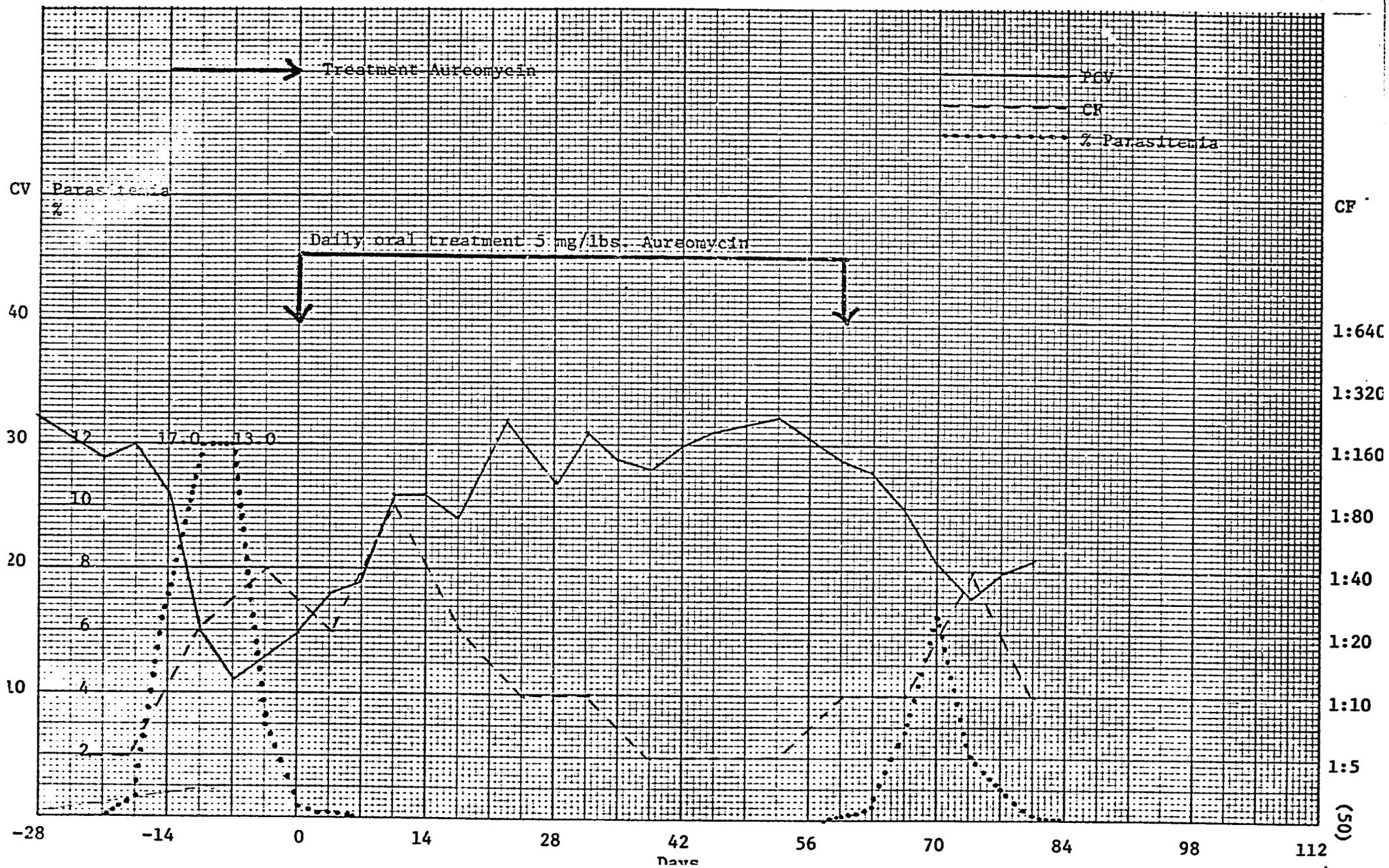
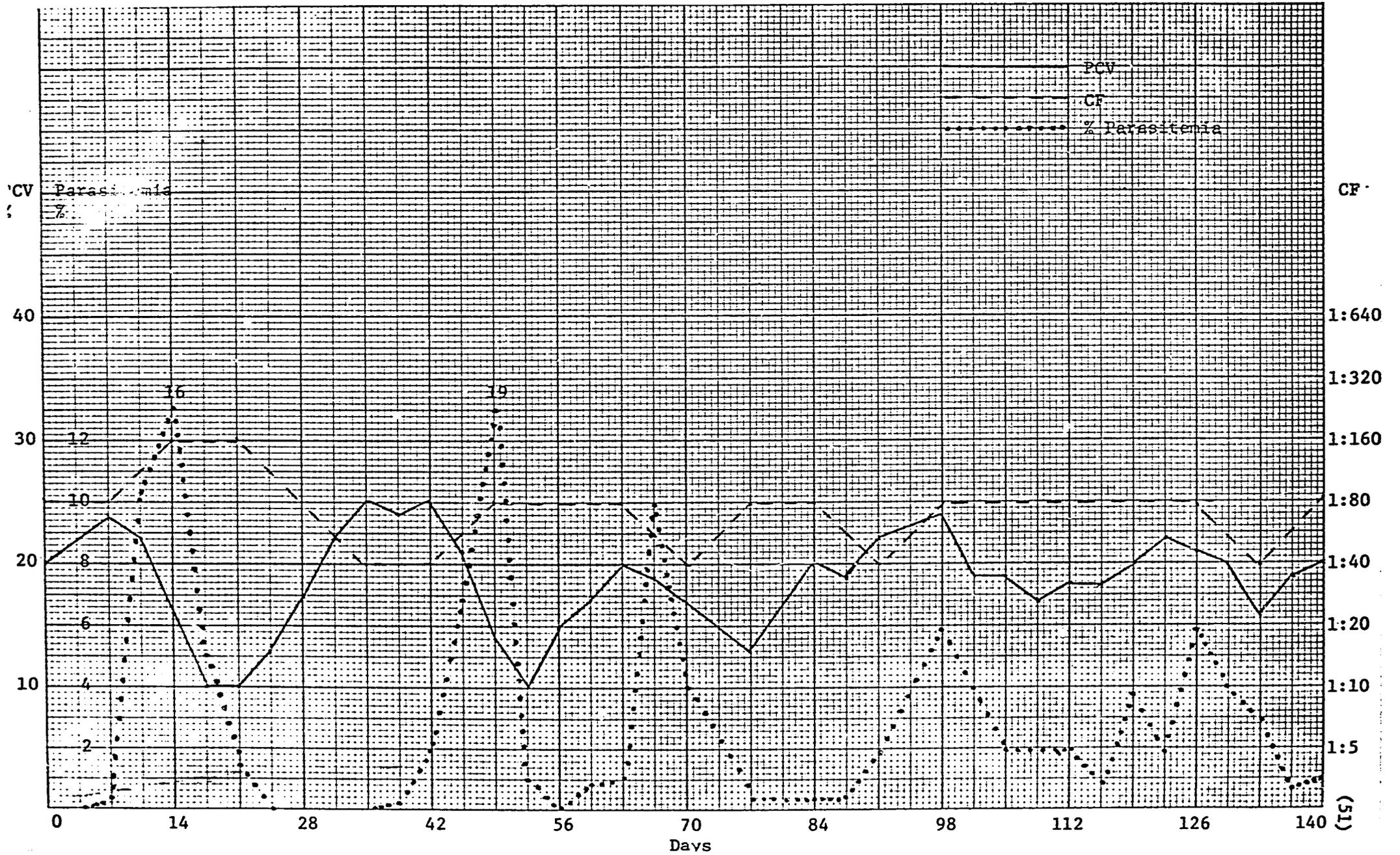


Figure 14 Calf 191  
 Control (for Calf 459 and 463)



Treatment Experiment #6:

We have successfully eliminated the Anaplasma carrier state in splenectomized calves by 3 inoculations of 4A65 at 24 and 48 hour intervals, and by 2 inoculations at a 2 week interval. It would be desirable in light of the 30 day cycle seen in the untreated control to measure the response to multiple treatment with 4A65 at a 28 day interval.

Calves 459 and 463 (figures 15 and 16), have been treated identically, insofar as treatment is concerned. Both are splenectomized carriers, and beginning on day 0 each was treated with 5 mg/kg 4A65 injected s/c. Such treatment consistently failed to prevent the Anaplasma cycle. Neither animal developed severe clinical signs of infection, the PCV's remained fairly constant when compared to the untreated control, indicating the marked influence of 4A65 in preventing severe Anaplasma response. In 463 this cycle occurred at a fairly consistent 30 day interval. Calf 459 showed a more erratic cycle pattern, but recurring parasitemias were noted. This pattern leads us to postulate that the drug is selective in its action, and that some phase of the Anaplasma life cycle is not susceptible, and that a resistant phase is developing during the absence of a detectable parasitemia which later produces a parasitemia regardless of previous treatments, and that treatment is effective once again only on the susceptible phase. There is no evidence of the development of drug tolerance on the part of the Anaplasma organism, but this monthly treatment is continuing to test this possibility. In addition observations including BUNs, and SGOTs will continue for possible accumulative toxic reactions to 4A65.

Figure 15 Calf 459

Treatment - 5 mg/kg - 4A65 - 4 week interval

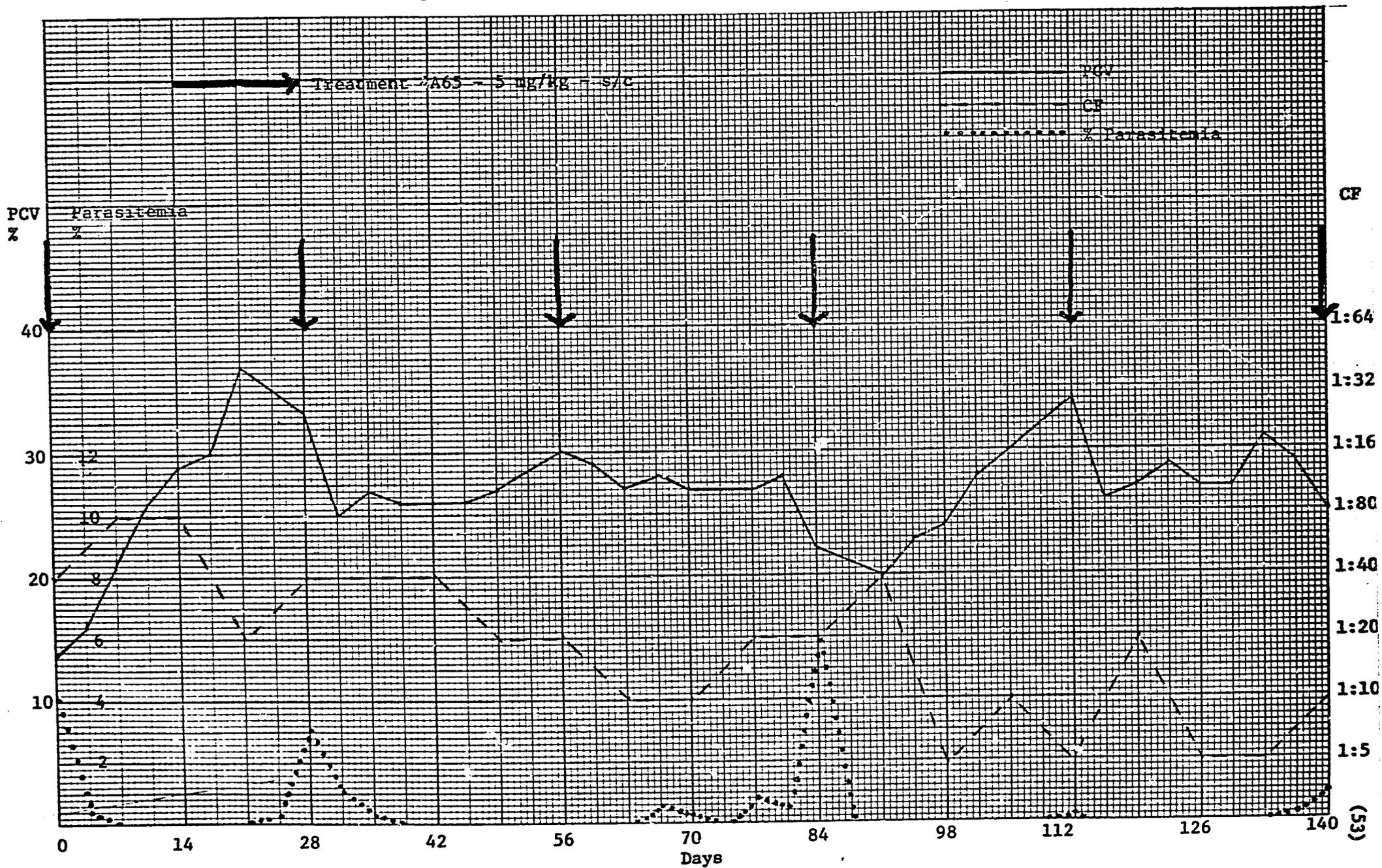
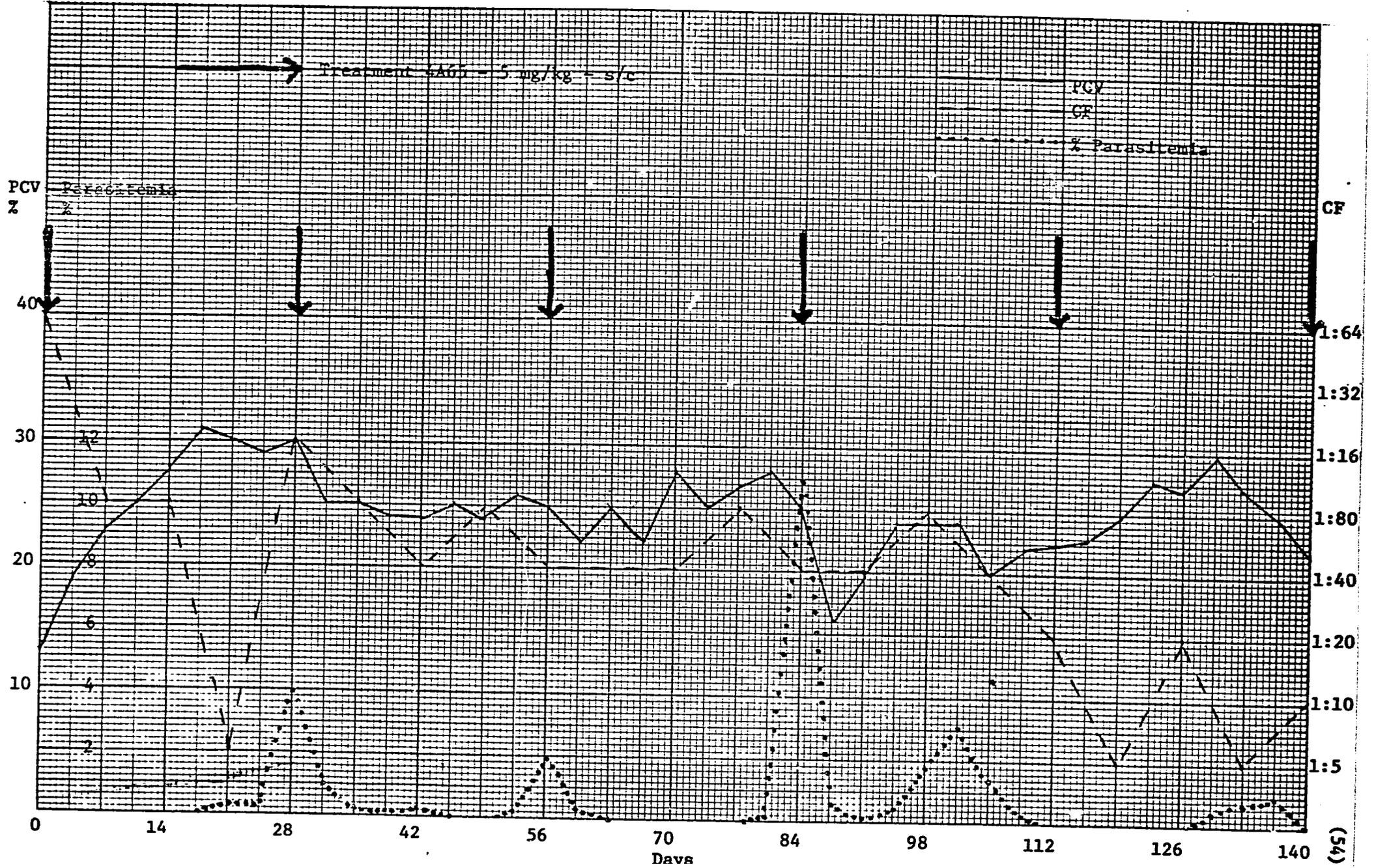


Figure 16 Calf 463

Treatment - 5 mg/kg - 4A65 - 4 week interval



Comparative Response of Premunization Using  
an Attenuated Anaplasma marginale, a Virulent A. marginale  
and A. centrale in Different Age Groups

(Submitted for Publication in Tropical Animal Health and Production)

Anaplasmosis immunization, in areas where this disease is endemic, has for years been accomplished by the technique of premunization. In young animals, because of natural resistance, it is possible to use fully virulent Anaplasma marginale to induce active infections and premunization under some conditions (Schmidt, 1937). In many endemic zones unintentional premunization is known to occur in a high percentage of calves as the result of transmission by natural vectors (Kuttler and Bohman, 1960). Even though such calves generally become resistant to future acute infections they remain carriers of the infection, thus perpetuating the disease in a given area. A second possible hazard associated with either intentional or unintentional premunization with virulent Anaplasma is the mild, transient, but sometimes persistent anemia associated with such infection. It is not known to what extent this stress factor may influence the animals ability to cope with other hemoparasitic diseases and internal or external parasitic infestations, which contribute an added burden on the hemopoietic system. The disease hazard of premunition with virulent A. marginale is increased in older animals, and is generally not recommended except where the disease can be moderated by specific drug therapy.

Theiler (1911), in his first description of A. centrale, suggested that premunization with this organism might be useful in preventing the more severe disease caused by A. marginale. Walker (1915), reported on the use of A. centrale as a vaccine for anaplasmosis in South Africa. He

reported that a prior infection of A. centrale reduced the severity of a later A. marginale infection. The serologic and immunologic relationships of A. marginale and A. centrale were described in greater detail by Kuttler (1967 a and b). There appeared to be common antigens present in the two organisms, which were characterized by cross reactions with the complement-fixation test. Animals premunized with either A. centrale or A. marginale resisted a later challenge by either the homologous or heterologous organism. The resistance to a homologous challenge was complete whereas the degree of resistance to the heterologous challenge was incomplete. This prompted the observation that an attenuated or mild A. marginale would offer better protection under those situations where premunition was indicated.

Ristic, Sibinovic, and Welter (1968) described the attenuation of A. marginale for cattle by numerous serial passages through sheep. The resulting organism was described as producing a mild infection, with few if any clinical manifestations of anaplasmosis, followed by complete protection against a virulent A. marginale (Welter and Woods, 1968). It was presumed that the attenuated organism would establish a carrier infection, which would result in a safe, long lasting immunity.

Kuttler, Zaraza, and Roberts (1968) basically confirmed the earlier reports of the mild nature of the attenuated organism, and the solid immunity to needle challenge using a virulent A. marginale of Texas origin. Field challenge on the north coast of Colombia, however, resulted in marked infections among calves vaccinated with the attenuated organism, suggesting the presence of an antigenic variant of A. marginale in this area (Zaraza and Kuttler, 1971).

The value of the attenuated A. marginale against antigenically similar, virulent organisms strongly suggests the use of this agent in areas where premunition is the method of choice for anaplasmosis control. The

severity of the premunizing infection assumes importance in the selection of an Anaplasma strain for this purpose. This study attempts to compare the relative response of adult cattle, splenectomized calves, and intact calves to premunizing Anaplasma infections induced by fully virulent A. marginale, the attenuated A. marginale and A. centrale.

Materials and Methods:

Infections using field isolates of fully virulent Anaplasma marginale (VAM), attenuated A. marginale (AAM), and A. centrale (AC) were induced in 46 mature cattle, 33 intact and 38 splenectomized calves.

The average age of 46 adult cattle used in these trials was 6 years ( $\pm 2.9$ ). They were European type cattle of mixed breeding. A total of 14 adult cattle received the attenuated A. marginale (AAM). Seven were injected subcutaneously (s/c) with 5 ml of vaccine\* (AAM), which had been stored frozen and was thawed immediately prior to use. The remaining cattle each were inoculated with 1 ml s/c of freshly collected whole blood from a splenectomized calf carrying a first bovine passage of the AAM organism. Fourteen cattle were injected s/c with 5 ml of blood freshly collected from a splenectomized calf carrying an AC infection.\*\* A VAM infection was induced in 18 cattle. Six cattle were infected by the s/c injection of 5 ml of whole blood freshly collected from a splenectomized carrier calf. The remaining 12 cattle in this group were injected s/c with 1 ml each of whole blood from a splenectomized carrier calf.

The average age of the 33 intact calves used in these trials was 3.6 months ( $\pm 2.3$ ). These calves were European dairy type cattle of mixed

\* Diamond Laboratories, Des Moines, Iowa.

\*\* Obtained from The Kenya Veterinary Department, Veterinary Laboratory at Kabete. They in turn had received the organisms from South Africa.



complement-fixation reaction or a 1% Anaplasma parasitemia. The duration of anemia was measured, in days, as that period when the PCV was 75% or less of the normal pre-infection PCV. The percent of normal PCV measures the relative drop in PCV as the result of Anaplasma infection taking into consideration the pre-infection PCV. The low PCV, high parasitemia, and the high CF titer observed during the course of infection are recorded in the tables of results.

Results:

The response of adult cattle premunized with AAM, AC, and VAM, in the absence of any treatment, is presented in Table 1. In nearly every category significantly more severe reactions occurred in animals infected with VAM, as compared to AAM or AC. There was no significant differences in the severity of infections caused by AAM and AC. A significantly lower CF titer was detected in those cattle having AC infections. No deaths occurred among the AAM and AC groups, but 2 cattle died in the VAM group.

The response of intact calves, premunized with AAM, AC, and VAM, in the absence of treatment, is presented in Table 2. A more severe response to VAM infection was observed, but with the exception of the duration of anemia these differences were not significant at the 0.05 level. Calves inoculated with AAM had a longer incubation time than in the other 2 groups. The duration of anemia, while showing considerable animal variation was significantly longer in VAM infections.

The response of splenectomized calves premunized with AAM, AC, and VAM, in the absence of treatment, is presented in Table 3. The reactions observed in these animals resembled more nearly the response of adult cattle. In nearly all categories significantly more severe reactions occurred in animals infected with VAM as compared to AAM or AC. Comparisons between AAM and AC showed a more severe reaction occurring in calves infected

with AC. Calves infected with AC had significantly higher parasitemias, and a relatively lower PCV. The incubation time of calves infected with AAM was significantly longer than experienced with either AC or VAM.

Discussion and Conclusions:

The longer incubation time observed in mature cattle and calves inoculated with AAM probably reflects the inoculum used to induce infection. The AAM inoculum in most cases, particularly in calves where differences reach significance, had been frozen. Bedell and Dimopoulos (1962) demonstrated an increased incubation time following freezing, but no significant alteration in severity of infection as manifested by parasitemia. Lotze (1947) and Kuttler (1966) made the observation that the number of infective particles or the inoculum size influenced the incubation time more markedly than the actual severity of infection.

The relatively mild response of intact calves to all 3 anaplasmas was anticipated and confirms the belief that young animals have a natural resistance to this organism. Of possible significance, however, is the persistence of the anemia resulting from VAM infections in all 3 age groups. In the absence of regular blood examinations, many of these cattle would have appeared normal, but in reality the PCV remained markedly below normal for an extended period of time. No attempts were made in these studies to determine the influence of this low PCV on weight gain, productivity, or susceptibility to other diseases, but it would appear that consideration should be given these factors. Natural premunition with virulent field strains are probably followed by a period of depressed PCVs.

The significantly lower CF titers associated with AC infection is probably a reflection of serologic differences between A. centrale and A. marginale as described by Kuttler (1967).

There is little doubt that the results presented in this study show an attenuated reaction following AC and AAM infections as compared to VAM. Among intact calves and adult cattle there appeared to be no detectable difference between AC and AAM, however in splenectomized calves the response to AC infection was more severe than that produced in AAM.

It would appear that AAM is at least equally as mild in its premunizing infection as is AC. The antigenic relationship of AAM to VAM is probably closer than that of AC to VAM. For this reason there would appear to be some marked advantages to using an AAM when premunization appears to be the method of choice in preventing clinical anaplasmosis.

Summary:

Premunizing infections using a virulent Anaplasma marginale (VAM), an attenuated A. marginale (AAM) and A. centrale (AC) have been induced in 46 mature cattle, 33 intact calves, and 38 splenectomized calves, for the purpose of comparing the relative animal response to these infections.

The VAM produced significantly more severe reactions in adult cattle, and splenectomized calves, and a slightly more severe response in intact calves; however, these animals were relatively more resistant to all 3 infections. There was no detectable difference between the reactions caused by AAM, and AC when measured in adult cattle and intact calves. Among splenectomized calves, however, the AAM infections resulted in a milder response as measured by the relative drop in packed cell volume and percent parasitemia. The CF response was significantly lower in the AC infection.

Acknowledgements:

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Table 1  
 Response of Adult Cattle to Premunition with Attenuated A. marginale,  
A. centrale and Virulent A. marginale  
 (Avg. age 6.04 ± 2.9)

	No. of Animals	Avg. Pre-Infect. PCV (%)	Avg. Incub. Time (Days)	Avg. Low PCV (%)	Avg. Percent of Normal Low PCV (%)	Avg. High Paras. (%)	Avg. High CF Serum Titers	Duration of Anemia (Days)	Deaths
<u>Attenuated A. marginale</u>	14	34.4 ± 4.8	25.1 ± 20.7	24.9	73.1	3.40	1:390	8.6	0
<u>A. centrale</u>	14	36.5 ± 3.6	17.1 ± 5.3	26.5	72.4	4.04	1:131*	8.3	0
<u>Virulent A. marginale</u>	18	32.9 ± 6.5	22.6 ± 6.5	14.5	43.7	12.10	1:470	26.6	2
Significance		NS	NS	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	
DRS				5.4	11.7	5.0	*Lower Than 1:470	11.6	

±: Standard deviation.

NS: Not significant.

DRS: Difference required for significance.

Table 2

Response of Intact Calves to Premunition with Attenuated A. marginale,  
A. centrale and Virulent A. marginale

	No. of Animals	Avg. Age Months	Avg. Pre-Infect. PCV (%)	Avg. Incub. Time (Days)	Avg. Low PCV (%)	Avg. Percent of Normal Low PCV (%)	Avg. High Paras. (%)	Avg. High CF Serum Titers	Duration of Anemia (Days)
<u>Attenuated A. marginale</u>	10	5.1 ± 3.0	31.0 ± 4.6	29.3	22.0 ± 4.6	70.8 ± 7.4	1.56 ± 2.1	1:211	6.4
<u>A. centrale</u>	6	2.3 ± 0.8	34.8 ± 3.5	10.8	23.5 ± 8.1	67.2 ± 21.0	5.60 ± 8.4	1:320	5.0
<u>Virulent A. marginale</u>	17	3.2 ± 1.8	33.1 ± 4.7	15.4	19.5 ± 4.4	59.3 ± 12.6	7.10 ± 14.9	1:295	25.5
Significance		NS	NS	P<0.01	NS	NS	NS	NS	P<0.05
DRS				6.7					19.0

±: Standard deviation.

NS: Not significant.

DRS: Difference required for significance.

Table 3

Response of Splenectomized Calves to Premunition with Attenuated A. marginale,A. centrale and Virulent A. marginale

	No. of Animals	Avg. Pre-Infect. PCV (%)	Avg. Incub. Time (Days)	Avg. Low PCV (%)	Avg. Percent of Normal Low PCV (%)	Avg. High Paras. (%)	Avg. High CF Serum Titers	Duration of Anemia (Days)	Deaths
<u>Attenuated A. marginale</u>	13	34.2 ± 5.7	27.4	17.8	53.3	7.3	1:675	21.0	0
<u>A. centrale</u>	9	36.4 ± 3.4	18.7	15.8	43.7	35.8	1:204*	14.6	0
<u>Virulent A. marginale</u>	16	36.8 ± 5.7	20.8	8.5	23.6	63.0	1:364	50.7	12
Significance		NS	P<0.05	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	
DRS			8.6	2.5	7.64	20.3	*Lower Than 1:675	11.6	

±: Standard deviation.

NS: Not significant.

DRS: Difference required for significance.

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## Characteristics of a Premunizing Infection in Adult Cattle

### Using the Attenuated A. marginale

Seven cows and 1 bull were used in these trials to evaluate the nature of the premunizing infection, induced 9 months previously with an attenuated A. marginale. All 8 animals were injected s/c with 5 ml of vaccine (obtained from Diamond Laboratories, Des Moines, Iowa) consisting of sheep blood carrying the attenuated A. marginale strain which had been stored frozen prior to inoculation. The reaction of these cattle to the premunizing infection is described elsewhere in this report (Comparative Response of Premunization Using an Attenuated Anaplasma marginale .....).

Very little information is available regarding the duration of immunity or premunity following infection with the attenuated organism even though it is assumed that it will resemble natural infection. If this is the case it might well be that premunity would persist essentially for the lifetime of the animal. The recognized attenuated nature of the infectious agent might well influence this persistence, and for this reason challenge of these cattle has been delayed for 9 months. Other animals will be challenged at even later dates.

It is not known which organism remains dominant when a virulent infection is superimposed on an attenuated infection. For this reason blood inoculations will be made into splenectomized calves from cows premunized with the attenuated organism and later challenged with virulent Anaplasma. Comparisons of the relative severity of such infections with calves inoculated with blood from cows carrying only the attenuated strain should indicate which organism remains dominant.

### Materials and Methods:

Four Hereford cows (Nos. 286, 289, 291, and 293) with an average age of  $8.75 \pm 1.5$  years, that had been premunized 9 months previously with the attenuated organism, were challenged with virulent A. marginale by injecting them with 1 ml freshly drawn blood from splenectomized Calf 135 having a 14% parasitemia and a PCV of 13%. These animals were observed 84 days for evidence of challenge infection. Complement-fixation tests were conducted on serum samples, parasitemias determined on Giemsa-stained thin blood smears, and PCV observed for evidence of challenge infection. Fifty days post challenge 100 ml of blood was drawn from Cow 286, and an equal amount from Cow 289. Splenectomized Calf 427 was inoculated I.V. with 100 ml of blood from 286, and splenectomized Calf 449 received blood from Cow 289.

Three Hereford cows and 1 Hereford bull (Nos. 287, 292, 295, and F73) with an average age of  $6.0 \pm 3.7$  years, that had been premunized 9 months previously with the attenuated organism, served as non-challenged controls. A transfer of 100 ml blood was made, on the same date as challenged cows, from Cow 287 and 292 into each of 2 splenectomized calves (446 and 451 respectively).

A yearling heifer, (297) not previously exposed to either the virulent or attenuated A. marginale, was challenged as a control at the same time, receiving the same inoculum as used to challenge Cows 286, 289, 291, and 293.

The same observations were made on the 4 splenectomized calves and 297 to characterize the severity of infection.

### Results:

Figure 1 shows the average PCVs of the challenge and non-challenge groups, for 84 days after challenge. No measurable increase in serum CF

titers or parasitemia were detected in either group. The PCV remained essentially the same in each group. The control (297) reacted severely to the same challenge with an incubation time of 10 days and a rapidly progressing anemia reaching a low of 11% on day 24. This low PCV was associated with a 6% parasitemia and a diagnostic CF response. The control (297) was not expendable so at day 24, to reduce the risk of fatal response, the animal was treated with 5 mg/kg 356 C 61 and 11 mg/kg oxy-tetracycline. Recovery following treatment was prompt.

The average PCV and parasitemia for Calves 427 and 449 are plotted in Figure 2; similar information for Calves 446 and 451 are recorded in Figure 3. Calves 427 and 449 responded severely to challenge from cows that had been challenged with virulent A. marginale. Calf 427 died on day 59. Calves 446 and 451 reacted equally as severe, even though the donor cows had been exposed only to the attenuated strain. Both calves died of acute anaplasmosis, death occurring on days 35 and 42.

#### Discussion and Conclusions:

A virulent A. marginale failed to produce evidence of anaplasmosis in 4 cattle premunized 9 months previously with the attenuated organism. There were no significant differences between the non-challenged and the challenge groups, even though the inoculum used for challenge was demonstrated to be fully pathogenic.

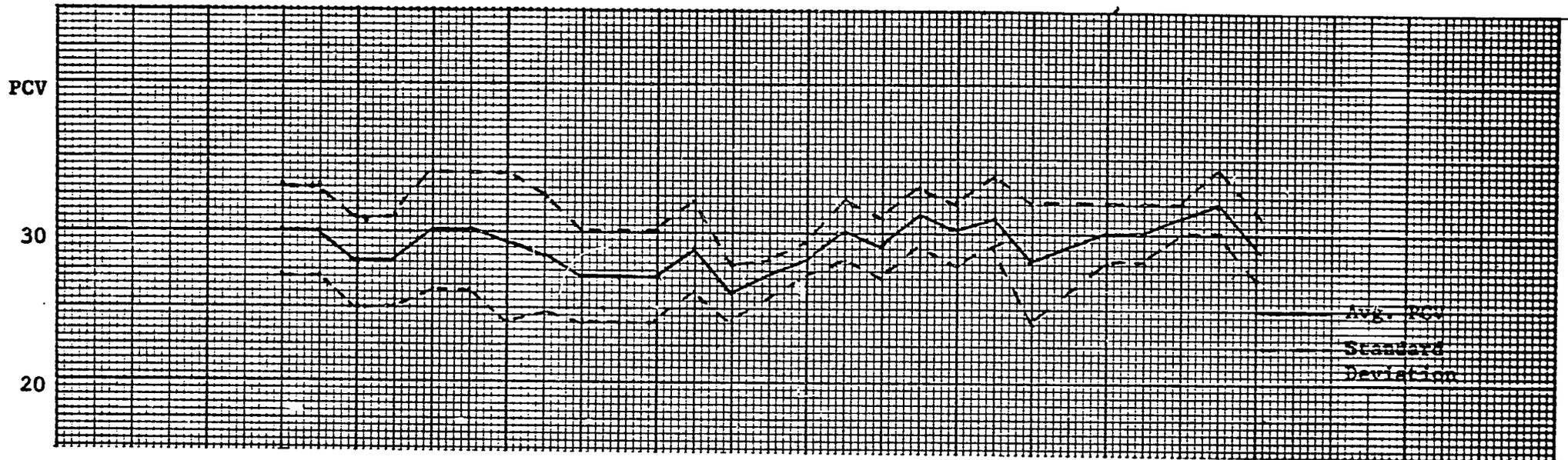
The Anaplasma organism recovered from 2 cows vaccinated about 11 months earlier with the attenuated A. marginale proved to be equally as virulent as the organism recovered from the challenge cattle. These results suggest that the Diamond organism may have reverted to virulence in the first bovine passage, over a period of time. This reversion has been demonstrated statistically by rapid back passages in splenectomized calves, but never under these circumstances. The large inoculum administered the

splenectomized calves may have influenced this reaction, but equal inoculums were used from cows supposedly carrying the virulent organism. Another possible explanation is that these cattle had been naturally exposed to virulent Anaplasma during the 11 month period between premunition and re-isolation of the organism in splenectomized calves. This seems unlikely since susceptible splenectomized calves were placed in the herd to detect possible natural transmission, (see 1971 annual report) and these calves failed to show any evidence of Anaplasma transmission of either the virulent or attenuated organism.

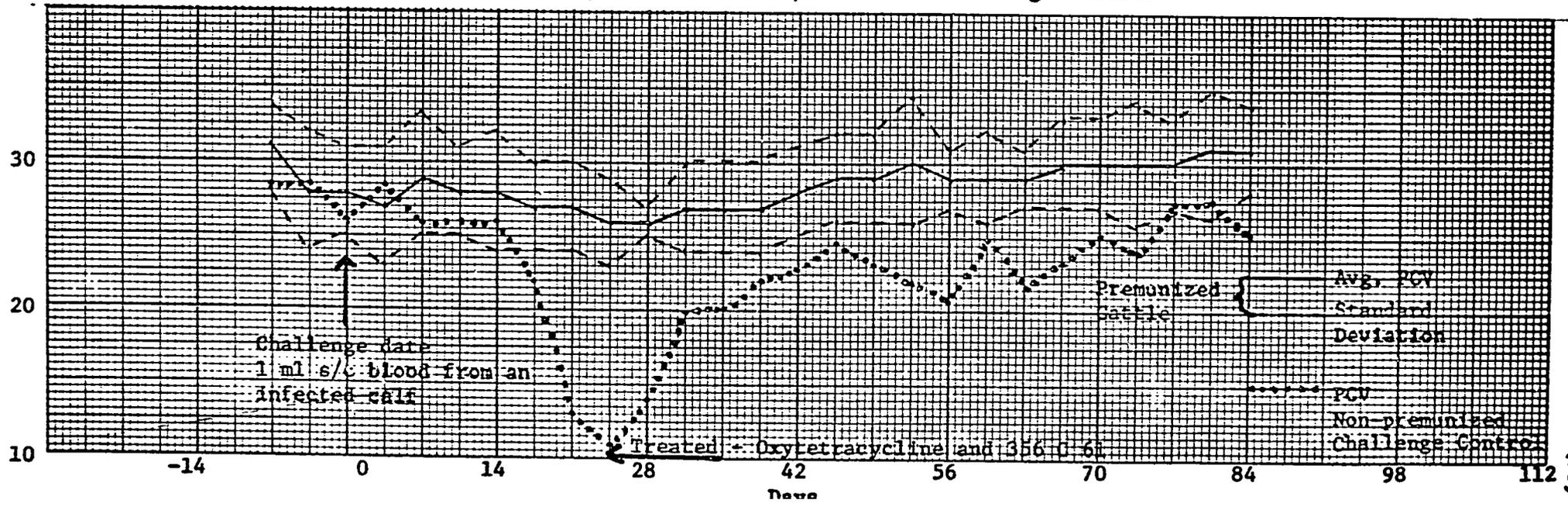
The reversion supposition must be further proved on more animals, and will be investigated subsequently as calves become available. If however these results are duplicated the implications of this finding will significantly influence the concept of premunition with an attenuated organism. First it seems highly unlikely that such an agent would have use in an area of possible eradication since vaccination would not only be perpetuating the disease, but would also eventually be maintaining the virulent organism. Secondly, however, the premunization with this organism in endemic areas might be expected to increase an animals protection against the virulent organism gradually as the organism reverts to its virulent phase. The premunized cattle show no adverse effects of this process since it likely occurs so gradually, but as time and this process continue the protection against virulent challenge might be expected to increase. Previous work has shown the Texas virulent organism to protect against even the Colombian strains, whereas the attenuated organism was less effective. With complete reversion of the attenuated organism a more complete protection could be expected. In the final analysis reversion to a virulent form may be advantageous in endemic zones since you have the advantage of a mild initial or acute phase of infection and the gradual reversion to an

organism comparable to the virulent A. marginale. Such an approach would eliminate to a large extent the high parasitemias associated with acute anaplasmosis, and thus reduce the likelihood of natural transmission. It is probable that transmission from the animals with an active parasitemia is more easily accomplished by natural vectors during the acute, high parasitemia phase than during the chronic or quiescent phase.

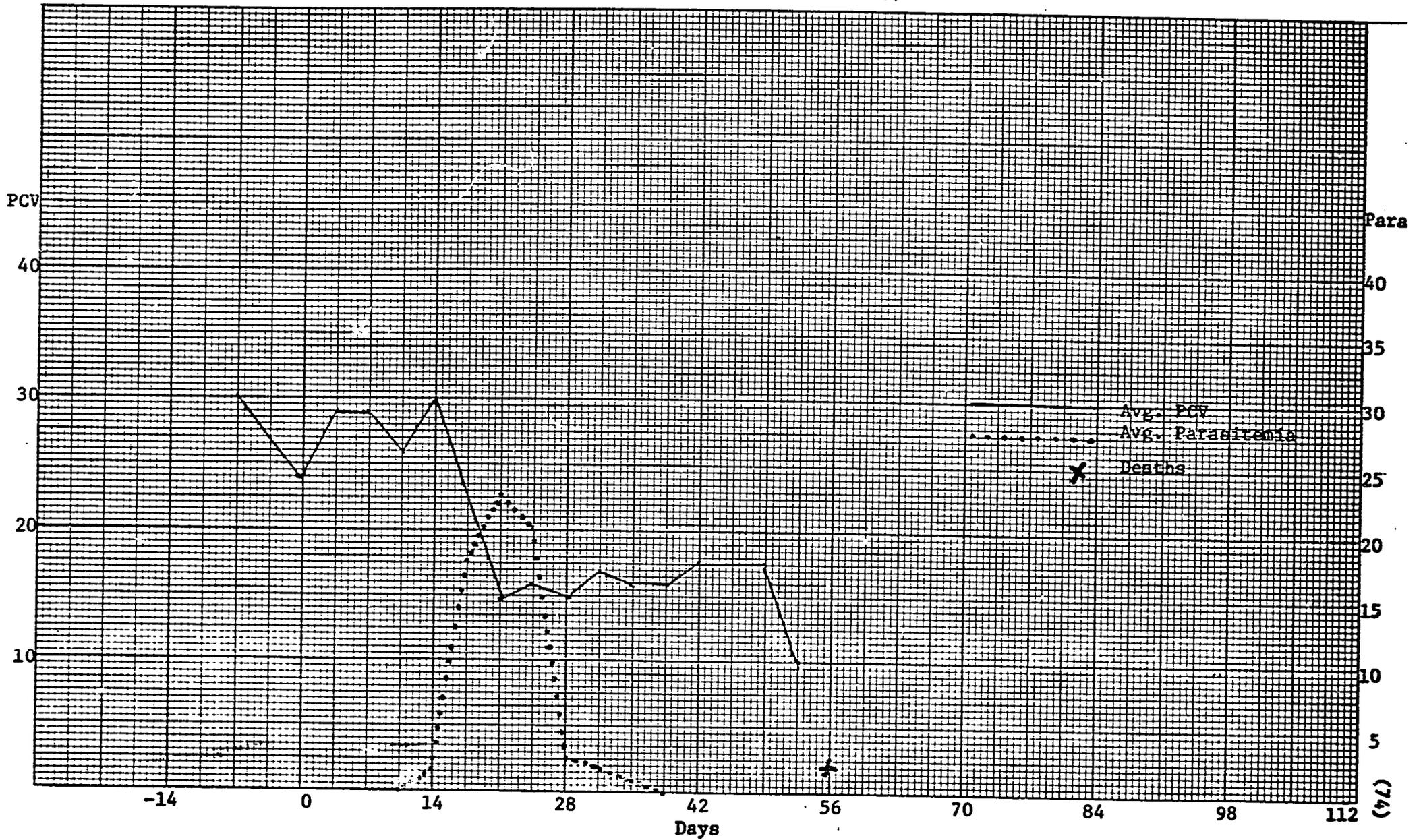
Figure 1  
 PCV Response  
 4 Premunized Cattle - Non-challenge - Controls



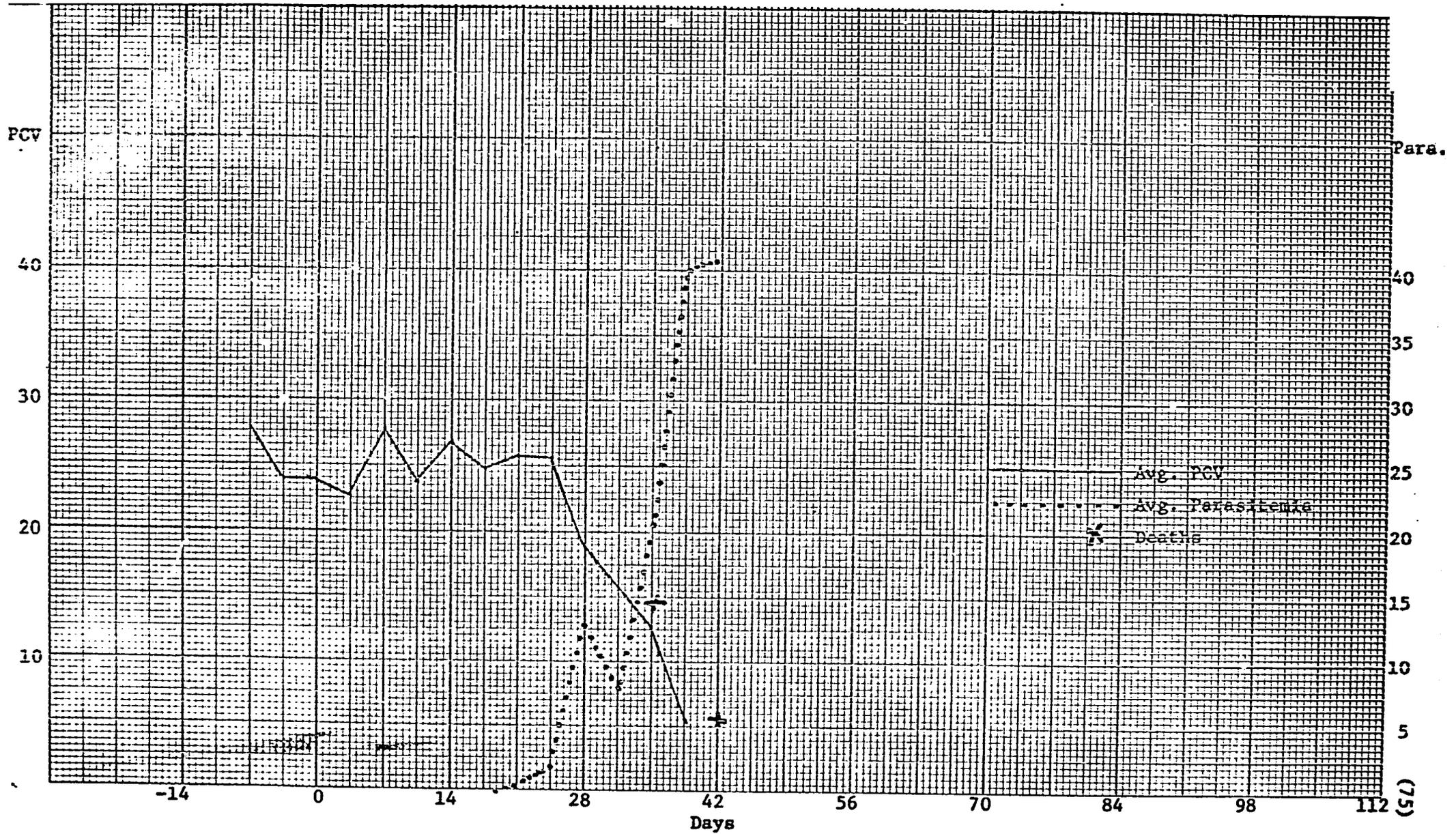
PCV Response  
 Challenge of 4 Premunized Cattle and 1 Challenge Control



**Figure 2**  
**PCV and Parasitemia**  
**Avg. Response of 2 Splenectomized Calves Inoculated With Blood From Cattle**  
**Recently Challenged With Virulent A. marginale**  
**Calves 427 and 449**



**Figure 3**  
**PCV and Parasitemia**  
**Response of 2 Splenectomized Calves Inoculated With Blood From 2 Cows, Each Infected With**  
**The Attenuated Strain**  
**Calves 446 and 451**



Anaplasma Antigen Studies

IV. Relative antigenicity of infected erythrocytes at varying stages of infection.

These studies are a continuation of work started in 1970 and included in the 1971 report. A basic problem is to obtain adequate numbers for valid statistical analysis. This report, as last years, should be considered preliminary in nature, and will be added to, before publication. We have, however, summarized available data for inclusion in this report.

Several variables in technique have been standardized in order to obtain more comparable results.

Antigens have been prepared basically by the same method as previously described for "Small Volume Antigen" (1971) but with the following standardizations. Efforts were made to obtain sufficient blood from each animal being tested to give at least 8 ml packed, washed erythrocytes. When this was impossible smaller volumes were used, but the reconstituted volume was altered so as to remain standard. Erythrocytes were washed 3 or 4 times in vernal buffer (VB), and packed cells (usually 8 ml) were lysed in 4 volumes of distilled water. Lysed erythrocytes were then frozen at  $-20^{\circ}\text{C}$ , and at a convenient time processed for antigen production. After thawing the lysed cells were centrifuged 40 minutes at 35,000 X-G. The sediment was re-suspended in distilled water by sonication, washed 2 times in distilled water, and 2 times in VB using the same centrifical forces. After the first VB wash the material was concentrated to 8 ml and centrifuged in a small tube. The sediment obtained after the second VB was resuspended in 8 ml VB using sonication to thoroughly break up

the sediment and obtain an even suspension. Corrections were made of the final volume in cases where less than 8 ml packed erythrocytes were harvested. If 7 ml packed erythrocytes were harvested, then the final volume was 7.0 ml. Generally 4-5 ml antigen was adequate for testing.

Antigen titrations were conducted in much the same manner as described in the 1971 report with minor modifications. An arbitrary dilution was selected on the basis of parasitemia, and changed if the results failed to show clear-cut end points. Low parasitemias usually reflect low antigenicity, so this factor was used to select an initial dilution. Titrations were then conducted using the following antigen amounts:

Tube	1	2	3	4	5	6
	0.1	0.2	0.3	0.4	0.5	0.6

Two rows of tubes were setup with identical quantities, using a 1:40 positive serum in one row, and a negative serum for control.

Readings were made on a Beckman "ACTA" spectrophotometer using the corresponding negative serum control as standard, and measuring the optical density at a wave length of 540 and slit width of 0.023. Based on serum and positive controls a percent fixation was calculated for each tube. The spectrophotometer takes both the control and test sample simultaneously and records the difference. A positive control is used to represent 100% fixation, and the negative serum control is used to measure the difference in each tube. A sample reading is as follows:

Tube	1	2	3	4	5	6	7
Undiluted Antigen Amt.	0.1	0.2	0.3	0.4	0.5	0.6	4+ Cont.
Acta Reading	0.00	0.019	0.122	0.303	0.544	0.614	0.726
% Fixation	0	03	17	42	75	84	100%

100% represents a 4+, or complete fixation. In this instance 100% or 1 unit was not obtained at the 6th tube. Using the percent fixation a

linear regression is plotted, and based on this a 100% end point is calculated and this considered as 1 unit. The following is a sample calculation:

"x" Antigen Amount	"y" % Fixation
.2	3
.3	17
.4	42
.5	75
.6	84
$\Sigma X$ 2.0	$\Sigma Y$ 221
$\bar{x}$ 0.4	$\bar{y}$ 44.2
$\Sigma X^2$ 0.9	
$\Sigma x^2 = \Sigma X^2 - (\Sigma X)^2/n = 0.1$	

$$\begin{aligned} \Sigma XY &= 442 \\ \Sigma xy &= \Sigma XY - (\Sigma X \cdot \Sigma Y/n) = 22.0 \\ b \text{ or Regression Coefficient} &= \Sigma xy / \Sigma x^2 = 220 \\ Y - \bar{y} &= b (X - \bar{x}) \text{ or } 100 - 42.2 = 220(x - .4) \\ x &= 0.66 \end{aligned}$$

Therefore 100% fixation would be achieved by 0.66 ml of undiluted antigen, which would be equal to 1 unit.

If one unit of undiluted antigen were present in 0.66 ml then 12.1 units would be present in 8 ml of antigen. All antigenic units recorded represent the total units in 8 ml packed RBC. This is an arbitrary volume and could have been expressed in any given amount, but because 8 ml was normally used it was adapted as a matter of convenience. If the antigen dilution had been 1:9 or 1:10 then the total number of units would have been 121.

In the 1971 report the relationship between parasitemia and total antigenicity of a given sample was demonstrated and found to be highly significant. The relationship on a total of 41 antigen titrations is plotted in Figure 1. These samples were from animals showing a wide variety of parasitemias without regard for the stage of infection. A regression coefficient of 7.227 was highly significant at a probability of error  $<0.01$  with a correlation coefficient of 0.926 which was equally significant.

The CF reaction of experimental antigens apparently provides an additional measure of the level of antigenicity, present at any particular time. The development of a successful killed vaccine is dependent on the accumulation and concentration of Anaplasma antigens. This can best be done by selecting the proper time for harvesting blood from the infected animal and to determine the amount of antigen and its concentrations in the final end product.

A group of 5 antigens from calves showing an early ascending infection, a group of 10 antigens from calves showing either a late ascending infection or a maximum parasitemia, and a group of 11 antigens from calves showing chronic infections were tested and compared to determine possible differences in antigenicity.

The results are tabulated in Table 1. The average parasitemias are obviously significantly different as are the average number of antigenic units which reflect the high and low parasitemias and the stage of infection. The relative antigenicity, however shows some differences and was calculated by the following formula:

$$\text{Number of Antigenic Units/Parasitemia} = \text{Relative Antigenicity (RA)}$$

The RA for early ascending infections and maximum infections was very nearly the same. The RA in chronic infections, however, was significantly greater ( $P < 0.05$ ) than either of the 2 other groups. The explanation for this may be due in part to the loss of sensitivity or due to technical difficulties in measuring the relatively small amounts being tested. If these results are valid they do suggest that the more mature Anaplasma bodies contain greater antigenic amounts for each body, or else there are antigens present which are not reflected by parasitemia.

Regression coefficients are calculated for each of the 3 groups and are recorded in Table 1, and plotted in Figure 2. It is of interest to

note that in the early phases of infection a corresponding increase of CF titer associated with parasitemia could not be demonstrated, in fact a negative value was obtained. There were however, only 5 samples in this group and this value was not significant. The late ascending or maximum parasitemia group showed a highly significant positive correlation between parasitemia and antigenic titer, with a regression coefficient (b) of 5.9. Antigens from chronically infected animals showed a somewhat higher value (b = 6.86) resulting in a steeper slope for this group in Figure 2.

Numbers are still inadequate to draw firm conclusions, concerning the dynamics of Anaplasma growth but continued studies will, it is hoped, give us a better understanding of the etiologic agent and the host - parasite relationship in anaplasmosis. This technique has shown promise as a measure of antigen which will be useful in immunologic procedures.

Antigen preparations using small volumes of blood will be studied from deer and sheep infected with Theileria, Babesia and Anaplasma infections to see if consistent correlations occur, and if relative antigenicity and - parasitemia - antigenicity relationships exist. In diseases where difficulties are experienced in finding the parasite, and before the development of serum antibodies such a test as described here might have diagnostic value.

Antigen titrations from sheep infected with anaplasmosis showed a relative antigenicity of  $10 \pm 5.3$  with a parasitemia of  $2 \pm 1.5$ , which is comparable to values in cattle of similar parasitemias.

**Table 1**

**Relative Anaplasma Antigenicity at Various Stages of Infection**

	<b>Early Ascending Infections</b>	<b>Late Ascending or Maximum Infection</b>	<b>Chronic Infections</b>	<b>Significance</b>
<b>No. of Observations</b>	5	10	11	-----
<b>Average Parasitemia</b>	5.6% ± 2.7	29.5 ± 12.0	1.2 ± 1.5	P<0.01 DRS= 9.4
<b>Average Antigenic Units</b>	29.8 ±10.5	222.7 ± 91.5	19.8 ± 15.7	P<0.01 DRS= 34.5
<b>Average Relative Antigenicity Amt. Units/Parasitemia</b>	6.4 ± 3.3	7.7 ± 1.96	18.3 ± 9.0	P<0.05 DRS= 8.1
<b>Regression Coefficient (Parasitemia X Antigenicity)</b>	-0.356 (NS)	5.9 (P<0.01)	6.86 (P<0.05)	-----

DRS - Difference required for significance.

Figure 1

## Correlation of CF Titers (Antigenic Units) and Parasitemia

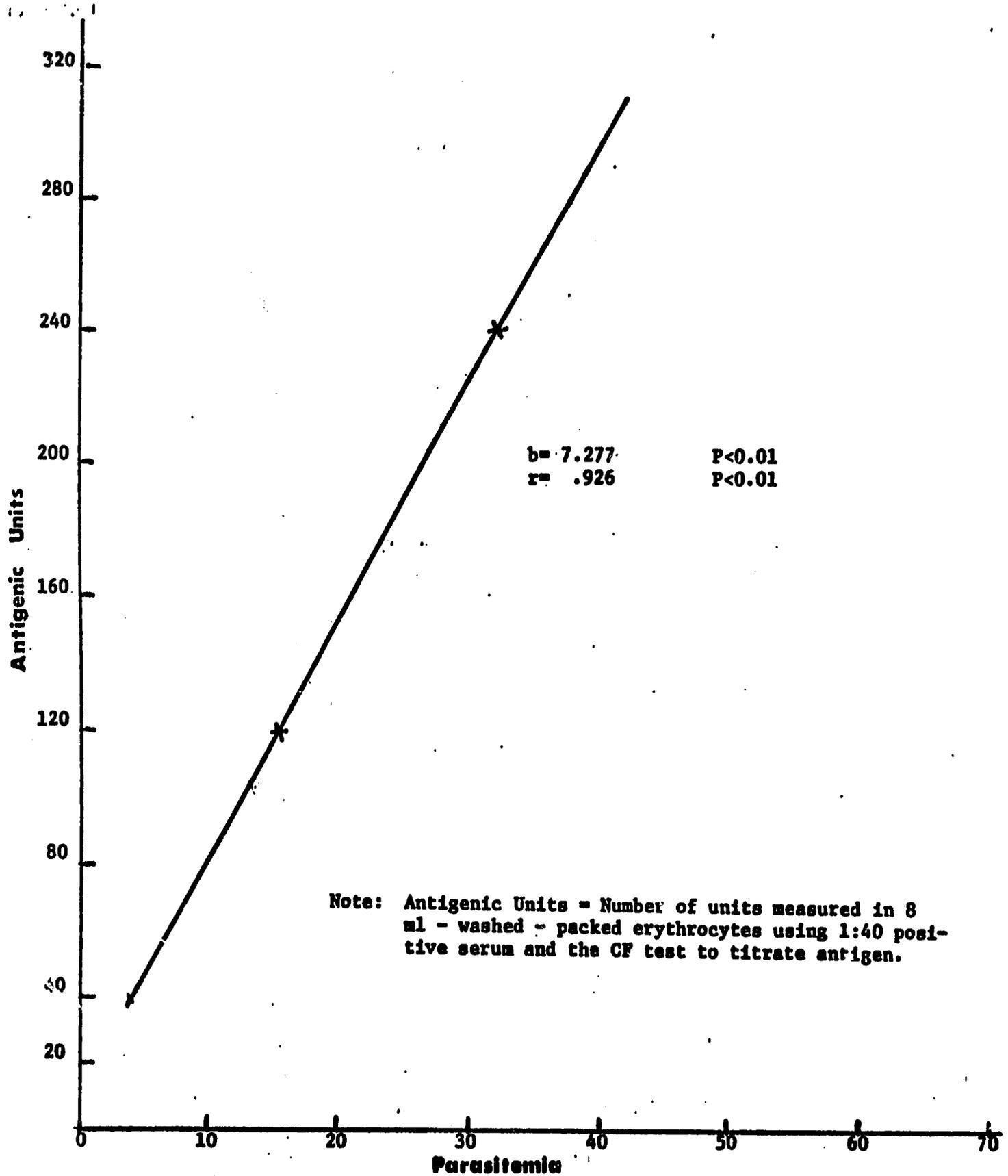
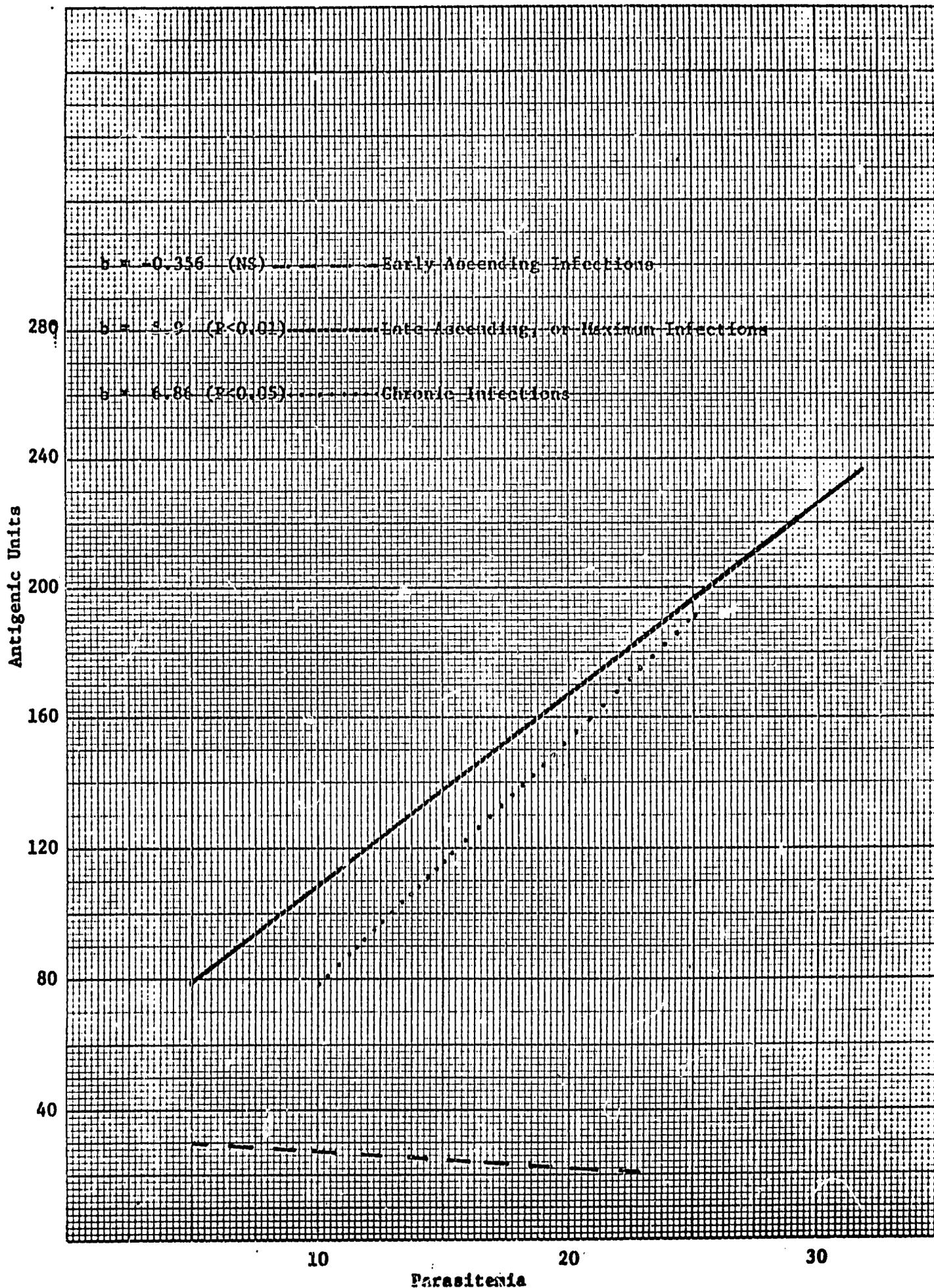


Figure 2  
Regression Coefficients Between Parasitemia and Antigenicity  
During Varying Stages of Infection

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### Anaplasmosis in a Lamb

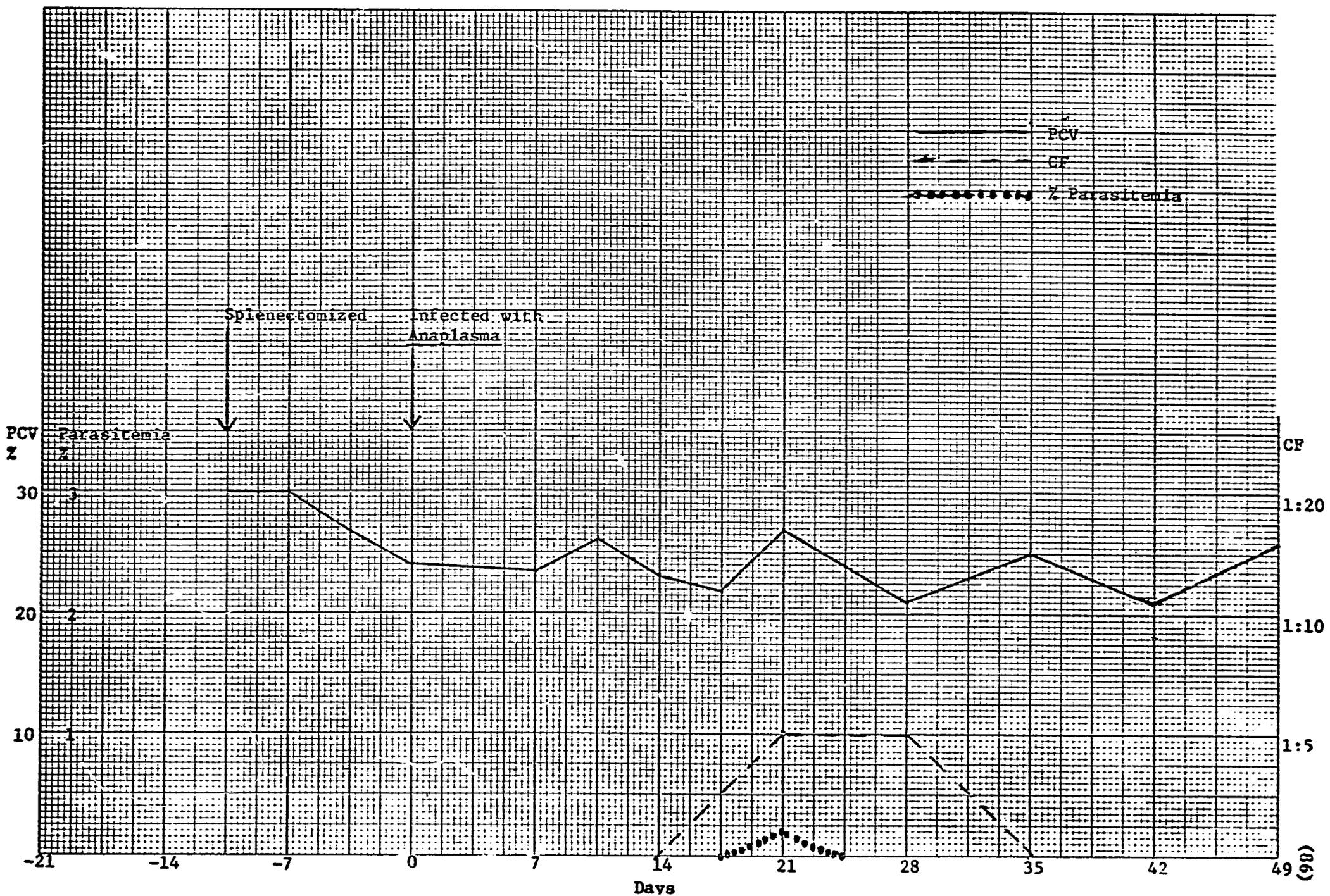
There is a need for a system or model whereby Anaplasma growth can be accomplished in small animals or in tissue culture. This is especially true in therapy trials where drug screens become desirable. Currently it is impossible to utilize a large number of compounds since all tests must be conducted on splenectomized calves, and these animals are not sufficiently numerous to use in this manner. Another drawback is the size of the experimental model. A calf by the time he can be used will weigh 55 to 75 kilograms. If a drug is to be screened at 10 mg/kg it means that 550 to 750 mg. would be needed for a single treatment. Currently drugs are being supplied in 100 mg. amounts, so it becomes obvious that markedly more drugs would be needed before screen trials would be accomplished in calves.

Anaplasma is known to grow and maintain itself in sheep, even though at a low level of activity. The attenuated strain of Anaplasma is supposedly adapted to sheep, so it was thought that the 8-10 pound (3.6 to 4.5 kg) baby lamb, might, if splenectomized provide a model in which growth could take place and where drug trials could be performed.

For this reason a baby lamb weighing about 7 pounds, was splenectomized at 7 days of age, and 11 days after splenectomy infected with Anaplasma marginale, the attenuated strain. A total of 10 ml of blood from Sheep 215, a carrier animal, was injected I.V. into this 18 day old splenectomized lamb. The results are shown in Figure 1. It will be seen that infection while obviously occurring was minimal, and would be of little practical value in the growth of A. marginale as an experimental model. A maximum CF titer of 1:5 was detected on day 21. This persisted 1 week and then disappeared. A 0.2% parasitemia was detected on day 21 which rapidly subsided.

The lamb withstood splenectomy well and developed normally notwithstanding surgery and infection with anaplasmosis. A year after this experiment this lamb is doing well, perhaps somewhat retarded insofar as growth is concerned.

### Anaplasma Infection in a Splenectomized Lamb



Horsefly Transmission of Anaplasmosis  
(Antonio Betancourt and K. L. Kuttler)

The generally accepted theory is that horsefly transmission of Anaplasma marginale is essentially mechanical in that the biting insect after feeding, moves from a carrier or acutely infected animal to a clean animal and actually transfers a small amount of blood. If transmission is to occur it is thought that the fly must move directly from the infected animal to the susceptible animal with no more than a few minutes interval. There is no evidence to suggest an Anaplasma life cycle in the fly, or to suggest that transmission is other than by mechanical means.

This study was designed to determine how long the flies (Tabanus sulcifrons and T. nigrovittatus) will remain infected after feeding on a known infected calf. To do this the flies were allowed to engorge and then after a measured time interval were ground and injected into a susceptible splenectomized calf.

Materials and Results:

Both the large T. sulcifrons and the smaller T. nigrovittatus were used. A feeding box with nylon mesh on one side was attached to the back or to the side of the calf with flies trapped inside. The flies were left in contact with the animal until feeding was accomplished. This was usually a lengthy procedure covering several hours in some instances. After feeding the flies were removed from the box, and after the designated time interval were ground in a Vir-Tis tissue homogenizer, and in some instances further disrupted by sonication. The coarse particles, wings, legs, etc., were removed by centrifugation. Susceptible, splenectomized calves were then injected with fly material via both the s/c and i/v routes.

A total of 7 splenectomized calves have been injected with fly suspension in efforts to recover the Anaplasma organism (Table 1). Calf 454 was injected with a suspension of 4 Tabanus nigrovittatus 30 minutes after feeding on a calf having a 5% A. marginale parasitemia. Infection occurred 29 days after inoculation and death due to acute anaplasmosis followed. Calf 245 was injected with a suspension made from one T. nigrovittatus 24 hours after feeding on a calf having a 15% parasitemia. Infection did not occur following this treatment. Calf 463 was injected with a suspension made from 5 T. nigrovittatus 24 hrs. after feeding on a calf having a 4% parasitemia. Infection was detected 31 days later which developed into acute anaplasmosis with final recovery. Calf 245A was injected with a suspension made from 3 T. nigrovittatus 48 hours after feeding on a calf having a 4% parasitemia. This calf failed to develop anaplasmosis. Calves 406 and 459 were each injected with suspensions made from 1 T. sulcifrons 30 minutes after feeding on calves showing a parasitemia of 15% and 7% respectively. Both calves developed acute anaplasmosis and Calf 406 died. The final calf used in these trials 458, was injected with a suspension made from 1 T. nigrovittatus 30 minutes after feeding on a non-infected calf. No response to this injection was detected.

A second experiment was conducted using 1 calf, in which transmission was attempted by allowing a fly previously infected to feed on a clean calf. Calf 455, was exposed to 3 T. nigrovittatus flies, using the box previously described. These flies had fed 2 and 8 days previously on calves with a 4% and 14% parasitemia respectively. No evidence of infection was encountered in Calf 455 by this procedure.

Conclusion:

We have not found evidence of a biological cycle in the fly, nor have we shown evidence to refute the belief that transmission is solely mechanical.

Evidence is presented to indicate a reasonably long survival time in the fly. It is probably not significant that the infection can be maintained 30 minutes in the fly, since a similar amount of blood maintained at room temperature or at 37°C would probably retain infection. The recovery of A. marginale 24 hours after ingestion by the fly does suggest conditions in the fly at least compatible with Anaplasma, and possibly could provide an environment allowing the Anaplasma not only to survive but to possibly grow. The presence of the organism in the fly for up to 30 minutes and 24 hours also suggests the possibility that the fly may not be limited to 3-4 minutes in which to transmit anaplasmosis after an infected blood meal.

**Table 1**  
**Transmission of Anaplasmosis by the Injection of Fly Tissue**

Calf <sup>1</sup>	Fly	No. of Flies Used	Parasitemia Donor Calf <sup>2</sup> %	Time After Feeding, Placed On Calf	Response of Recipient Calf				
					Incubation	Low PCV	High Paras.	High CF	Result of Infection
454	<u>T. nigrovittatus</u>	4	5	30 minutes	29 days	9	72	1:40	Died
245	<u>T. nigrovittatus</u>	1	15	24 hours	Negative - but susceptible to challenge				
463	<u>T. nigrovittatus</u>	5	4	24 hours	31 days	6	51	1:640	Recovered
245A	<u>T. nigrovittatus</u>	3	4	48 hours	Negative - but susceptible to challenge				
406	<u>T. sulcifrons</u>	1	15	30 minutes	18 days	9	14	1:20	Died
459	<u>T. sulcifrons</u>	1	7	30 minutes	21 days	6	34	1:80	Recovered
458	<u>T. nigrovittatus</u>	2	Normal Calf	30 minutes	Negative - but susceptible to challenge				

<sup>1</sup> Receiving the inoculation.

<sup>2</sup> Parasitemia of the calf at the time flies were feeding.

### Trypanosomiasis

This work has been limited at our laboratory but is being more actively pursued in The Department of Parasitology under the direction of Dr. T. J. Galvin and Dr. Antonio Betancourt. The work in Parasitology has been prepared by Dr. Betancourt as a Masters Thesis entitled "Studies on Transmission and Incidence of Trypanosoma theileri, Laveran 1902", an abstract of which is attached to this report.

Our work has been confined to making serial passages in blood agar, embryonated chicken eggs, in tissue culture media, one unsuccessful attempt at antigen production, and a field isolation in a splenectomized calf.

A total of 64 serial passages of T. theileri were made on blood agar before being discontinued. The original isolate was made in June 1970, and the final culture 2 December 1971, covering a period of 18 months. The cultures were made every week at a 7 day interval, being incubated at 28 to 30°C. The 64 passages over an 18 month period averages 1 passage every 8.55 days, which reflects the extension of the usual 7 day interval on a number of occasions. The Trypanosoma culture was lost after 64 passages. The media used for this prolonged growth was prepared essentially as described by Dr. E. A. Wells as follows:

(1) Blood agar base, Tryptose	32.0 gm.
(2) Dextrose (analytical grade)	1.6 gm.
(3) Distilled Water	800.0 ml.
(4) Sheep blood - defibrinated	200.0 ml.
(5) Penicillin (400 u/ml.)	400,000.0 units
(6) Streptomycin (0.5 mg/ml.)	500.0 mg.

The agar and dextrose were dissolved in distilled H<sub>2</sub>O and autoclaved at 120°C for 15 minutes. It was cooled to 50°C, and blood which had been collected aseptically and defibrinated with glass beads was added to the agar while stirring. The temperature at 50°C was maintained and using an automatic pipette the blood agar was dispensed into tubes, which were tilted until media hardened and this was stored at 5°C till used.

Egg Passage (Antonio Betancourt):

On 3 occasions embryonated eggs have been inoculated with Trypanosoma theileri to determine if growth will occur in this media.

Feb. 2, 1972: 12-10 day old embryonated eggs were used. The inoculum consisted of 0.1 ml washings from a first passage blood agar slant rich in trypanosomes. Half of the eggs were inoculated in the yolk sac (YS), half in the allantoic cavity (AC). On the fifth day after inoculation, at 37°C, all embryos were checked for evidence of growth. No embryo deaths were recorded. Trypanosomes were found in 3 of 6 eggs inoculated via the YS, and 3 of 6 eggs inoculated via the AC. The trypanosomes were observed in the allantoic fluids and in the blood of the embryos.

Feb. 9, 1972: 12-10 day old embryonated eggs were inoculated with 0.1 ml in the AC, using trypanosomes from a second blood agar passage. Seven of the eggs showed signs of bacterial contamination as evidenced by the death of the embryo. The remaining eggs were checked for trypanosome growth on day 4 and day 5. In no instance was trypanosome growth detected in any of the surviving embryos.

Feb. 23, 1972: 12-12 day old embryonated eggs were inoculated with 0.1 ml in the AC, using 6 day old trypanosome cultures from a fourth blood agar passage. These eggs were not checked for 7 days, and all proved negative for trypanosomes. Some of the chicks were about ready to hatch.

The partial success in the first effort indicates the need to further explore this method of cultivation. It was hoped that a predominance of trypanosome forms could be obtained by this method, which might in turn be transmissible to splenectomized calves for more detailed study. This work will be pursued as time permits.

Isolation in a Splenectomized Calf:

In the 1971 report efforts to isolate trypanosomes in splenectomized calves was reported. These results were discouraging in that no demonstrable parasitemia was observed, and no evidence of pathogenicity was seen. Cultures made from these calves were positive sporadically and then went negative.

On March 13, 1972 observations of a stained blood smear from a cow suspected of having anaplasmosis revealed the presence of a fairly large number of large trypanosomes identified as T. theileri. A day later 50 ml of blood was collected from this cow, located near Madisonville, and injected intravenously into splenectomized Calf 130. Blood cultures were made daily, and later weekly, on blood agar slants for evidence of trypanosome infection. The results are tabulated in Table 1, and show positive growth after day 6, which has remained positive for over 30 days. Cultures are continuing beyond this point to determine how long they will remain positive. No pathologic changes have occurred in Calf 130 which can be attributed to trypanosomes. A drop in PCV followed an episode of Eperythrozoon infection which was controlled by treatment with Neorasephenamine. Neorasephenamine was administered on 4-7-72. Positive trypanosome cultures have been obtained on 4-5, 4-10, 4-17, and 4-24, so it is unlikely that this treatment influenced the trypanosome.

Even though a parasitemia in Calf 130 has not reached a level sufficient to be seen, the frequency of positive cultures is encouraging.

Table 1

Trypanosoma theileri isolation in a Splenectomized Calf

Exposure Took Place March 14 With 50 ml Blood I.V.

Date Cultured	Day	Date Read	Day	Results Pos/Neg.
3-15-72	1	3-20-72	5	0/3
3-16-72	2	3-21-72	10	0/3
3-17-72	3	3-22-72	15	0/3
3-20-72	6	3-24-72	19	2/3
3-22-72	8	3-27-72	13	4/6
3-27-72	13	3-30-72	16	0/3
3-30-72	16	4-4-72	21	0/3
4-5-72	22	4-10-72	27	3/3
4-10-72	27	4-14-72	31	3/3
4-17-72	34	4-21-72	38	3/3
4-24-72	41	4-28-72	45	3/3

## Tissue Culture Media - for Trypanosome Cultures

(Ronnie Ragsdale)

There would be some advantage, from the point of view of antigen production, to grow trypanosomes in a media free of RBC. For this reason tissue culture media was prepared for use in culturing trypanosomes and to make serial passage. The following media was used:

Earle's Medium prepared as follows:

Solution 1 - EBS 10X	100 ml
Solution 2 - LAH 10X	100 ml
Solution 3 - NaHCO <sub>3</sub>	10 ml
H <sub>2</sub> O, sterile, ion exchange	790 ml
	1000 ml

Solutions combined and 10% sterile calf serum added.

Solution 1 - Modified Earle's Balanced Salt Solution 10X (EBS)

NaCl	80.0 gm
KCl	4.0 gm
Ca Cl <sub>2</sub>	2.0 gm
MgSO <sub>4</sub> ·H <sub>2</sub> O	2.0 gm
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	1.4 gm
Dextrose	10.0 gm
Phenol Red (1% Solution)	9.0 ml
Pen/Strep	2.0 ml
H <sub>2</sub> O, ion exchange q.s.	1 liter

Solution 2 - Lactalbumin Hydrolysate (LAH)

Lactalbumin Hydrolysate	50 gm
Distilled Water	1 liter

## Solution 3 - Sodium Bicarbonate 7.5%

Sodium Bicarbonate	7.5 gm
H <sub>2</sub> O, ion exchange	100 ml

All solutions were sterilized by Seitz filter. Solutions 1 and 2 were stored at -20°C. Solution 3 was stored at 4°C.

The media was dispensed in 10 ml quantities in sterile containers. Cultures were incubated at 28°C.

Passage #1: 0.5 ml whole, heparinized blood from Cow 293 was inoculated into each of 5 tubes on 1-25-72. Tubes were read on 2-1-72.

Tube No. 1	++
Tube No. 2	+
Tube No. 3	-
Tube No. 4	+++
Tube No. 5	+

Passage #2: 0.5 ml from Tube #4 was inoculated into each of 6 tubes on 2-1-72. Tubes were read on 2-8-72.

Tube No. 1	++
Tube No. 2	+
Tube No. 3	+
Tube No. 4	-
Tube No. 5	+++
Tube No. 6	+

Passage #3: 0.5 ml from Tube #5 was inoculated into each of 6 tubes on 2-8-72. Tubes were read on 2-15-72.

Tube No. 1	+
Tube No. 2	++
Tube No. 3	-
Tube No. 4	+++

Tube No. 5 -

Tube No. 6 ++

Passage #4: 0.5 ml from Tube #4 was inoculated into each of 6 tubes on 2-15-72. Tubes were read on 2-22-72.

Tube No. 1 ++

Tube No. 2 +

Tube No. 3 +

Tube No. 4 -

Tube No. 5 -

Tube No. 6 ++

The viability of trypanosomes under these conditions was gradually declining, and passage at this point was discontinued. Further work is planned to determine how many serial passages are possible, and what concentrations of trypanosomes can be achieved by this method.

One attempt at producing an antigen from the cultured trypanosomes was unsuccessful. Future attempts will be made.

## ABSTRACT

Studies on Transmission and Incidence of  
Trypanosoma theileri, Laveran 1902. (May 1972)

Antonio Betancourt Echeverry, D.V.M.

Directed by: Dr. Thomas J. Galvin

A total of 129 Tabanus nigrovittatus Macquart were collected and dissected 5 days after having fed on a cow infected with Trypanosoma theileri; flagellates resembling the genus Blastocrithidia were found in the hind gut of 8 of the Tabanidae. The possibility of these flagellates being the insect stage of T. theileri was suggested, but definite proof of this fact could not be obtained since inoculations into calves raised free from Trypanosomes failed to produce any infection. The need for further research on this area was stressed.

Studies on the incidence of infection with T. theileri in 235 cattle of different age, breed and sex, in 6 vegetational areas of Texas, were carried out. T. theileri was found in 149 (63.4%) of the 235 blood samples examined by culture in blood agar. The highest rates of infection were found in two localities (Angleton and Sugarland) close to West Galveston Bay within the Gulf Prairies and Marshes; a high incidence was also recorded at Sonora located on the Edwards Plateau.

The percentage of infection among calves less than 1 year old was much lower (18.4%) than that of the adults (80.5%). No significant difference was found between percentages of infection in adult males and females, nor between cattle of 3 different breed-groups (Holstein, Hereford and Hereford X Brahaman).

Babesia rodhaini: Drug Screen Trials

A series of drug trials have been conducted, using experimental compounds obtained from The Walter Reed Army Medical Center, Medicinal Chemistry Division as well as materials available commercially and obtained by special arrangement from cooperating drug houses.

Even though drugs are available which show considerable activity against Babesia, more effective agents would be highly desirable. The treatment of sick cattle and the alleviation of clinical symptoms is really a minor part of the potential for drug therapy in the control of hemotropic diseases. An agent which would eliminate the blood parasite from both the vertebrate and invertebrate hosts would find wide application. A program of prevention, and or gradual premunition using drugs offers a far greater application of these agents than the treatment of sick animals. A drug given once every 2 to 4 months, which would prevent clinical manifestations of the Babesia and Anaplasma infection would probably find wide acceptance in areas where these pathogens are endemic. The eradication of disease through therapeutic means is not impossible.

Babesia rodhaini while different, morphologically, from the cattle Babesias is related, and responds favorably to known babesiacidal agents such as Ganaseg and Imidocarb (see 1971 report). It is extremely difficult to screen drugs in cattle or other large animals for obvious reasons, but the mouse adapted Babesia is well suited for this function. This approach is not intended to establish drugs as useful therapeutic agents in the treatment of cattle, but rather to explore various chemical compounds for possible activity in the hope of finding patterns which will

direct our attention to classes of compounds which show promise for the future. A secondary objective is to explore various treatment regimes using combined therapy, slow release techniques, various time intervals, etc. to develop a better understanding of therapeutic problems. The compound 4A65 is active against both Anaplasma and Babesia rodhaini. Agents that prove effective against B. rodhaini will be tested against Anaplasma, so to a degree these trials have implications for Anaplasma.

Preliminary screen trials have been conducted on approximately 19 materials. The dosage and treatment regimes are tabulated in Table 1. An identification of each drug is given in Table 2.

#### Materials and Methods:

White mice, weighing approximately 30 grams have been used in these trials. All drugs have been tested in groups of 5 mice. Treatment schedule and drug dosage are indicated in Table 1. On day 0 all mice were challenged with B. rodhaini infected mouse blood by intraperitoneal (i/p) injections. The inoculums were standardized on the basis of percent parasitemia and packed cell volume (PCV). The mean corpuscular volume (MCV) was determined for mouse blood by making red cell counts, and comparing this with the PCV. Using percent parasitemia, PCV, and MCV an estimation of the number of infected red cells is possible. The challenge is expressed as the number of infected red cells injected and is presented in Table 1 for each challenge.

The drugs or treatment being tested was in most instances administered on day 0 and 4 in relation to the challenge time. In early trials the drug was given on day -4 to evaluate possible prophylactic value. This was discontinued as a part of the initial screening procedure to facilitate the testing of larger numbers of compounds with the idea that

such an approach would become a part of the testing procedures where activity was present.

The dosage used for each drug is indicated in Table 1, and with few exceptions is based on body weight. Freund's complete adjuvant (FCA) was arbitrarily given in 0.1 ml amounts i/p. Peanut oil was given in 0.6 ml amounts i/p, and 0.1 - 0.2 ml amounts subcutaneously s/c. Dimethyl sulfoxide was given in 0.1 ml amounts s/c. The dose of Poly I/C was expressed in micrograms (ug) given each mouse. In all other instances the drug dosage was standardized by mixing a known weight of the compound with sterile peanut oil or other diluent if indicated and administered on the basis of mg/kg body weight. On unknown drugs the usual range was 20 and 40 mg/kg given at 0 and 4 days, so that each drug was tested on a total of 20 mice. Where activity was noted as in the case of 4A65 and AG 74492 the dose range was altered as indicated.

The challenge was standardized to generally give a marked parasitemia on day 4. Day 0 was generally a Thursday with parasitemias being determined on days 4-5-6-7-and 8 beginning the following Monday. Thin blood smears were made on each mouse by clipping the tail to obtain a drop of blood. The smears were stained with Giemsa and read in the usual manner, determining the percent erythrocytes having one or more Babesia bodies. Deaths were recorded daily. A regression analysis was made on each mouse, usually on days 4-5-6-7-and 8 to obtain a regression coefficient (b) (as determined in Snedecor's "Statistical Methods"), which was then used to calculate the "Therapeutic Index" and to conduct an analysis of variance test for significance. The Therapeutic Index was calculated as follows:

$$\frac{\text{Untreated Controls: No. of Deaths} \times 10 + b}{\text{Treated Mice: No. of Deaths} \times 10 + b}$$

The usual groups contained 5 mice. As an example suppose 4 of 5 mice

died in the control group showing an average progressive parasitemia (b) of 24.92. In the treated group only 2 of 5 mice died, and the treatment resulted in a slower progressive parasitemia which averaged 15.44. Then

$$\frac{4 \times 10 + 24.92}{2 \times 10 + 15.44} = \text{Therapeutic Index} = 1.83$$

Significance is measured by an analysis of variance of the b values obtained for each of the treated and untreated controls. Each group of 5 treated mice was compared to a set of 5 untreated control mice, both groups receiving the same challenge inoculum.

Notwithstanding an attempt to standard challenge, considerable variation in parasitemia at day 4 was observed, making it essential that all comparisons be related to the appropriate controls. Standard deviations are expressed for each progressive parasitemia to facilitate comparison between treatments since only the treated versus controls were subjected to statistical comparisons, these being expressed in Table 1.

#### Discussion and Results:

Poly I/C was included in these trials because it has been demonstrated to stimulate interferon production. Interferon, while non-specific in nature, does retard the growth of many intracellular microorganisms, principally the viruses. Babesia and Anaplasma, also intracellular microorganisms resemble this pattern, so it was thought advisable to investigate this possibility. No measurable therapeutic index was observed following treatment with 200 ug on days -4, 0 and 4. Additional trials using larger dosages will be made, as well as in combination with known effective products for the possible presence of therapeutically enhancing capabilities.

Freunds complete adjuvant (FCA) was used not so much as a therapeutic agent but as a control since this agent may have use in future trials where slow drug release will be tried. In this instance it was desirable to

show that it had no activity in and of itself. The i/p injection of this drug 4 days before challenge produced an interesting response in that highly significant reduction in the progressive parasitemia was detected. The irritating nature of this compound is presumed to have stimulated an influx of inflammatory cells into the peritoneal cavity, which were then capable of retarding the rate of Babesia invasion, when the challenge was given i/p 4 days later. In another instance where peanut oil was given i/p a similar effect was noted, except that in this instance the effect was most noticeable when the oil was given i/p at approximately the same time as the i/p challenge was administered.

Peanut oil had no moderating effects on the progressive parasitemia when given 4 days before and 4 days after challenge. The group receiving peanut oil 4 days after challenge actually showed a significantly more severe reaction. The apparent mechanical and non-specific influence encountered with i/p injections of drugs suggested that the s/c route would be preferable. The s/c route is now used entirely for drug administration with the i/p route reserved for challenge. The hazard encountered using the s/c route is that in some instances leakage may occur after the injection. Trials to date however have not shown this to excessively affect our results.

Compound AB 34313, a tetracycline, showed a measure of activity when administered 4 days after exposure at the rate of 20 mg/kg. This same drug failed to produce significant alteration in the progressive parasitemia when given at the 40 mg/kg level, even though a marked increase in the therapeutic index was noted.

The drug 4A65 was shown to be highly effective against B. rodhaini in the following amounts: 10 mg/kg, 5 mg/kg, 2 mg/kg and 1 mg/kg. Some activity was noted at 0.5 mg/kg but this was near the end point. No significant activity was detectable at 0.25 mg/kg. Early work on 4A65 was with

a dihydrochloride formulation. More recent supplies have been formulated using a dipropionate salt. There is probably no reason to assume any change or alteration in efficacy between these 2 formulations, but it was thought desirable to compare their relative efficacy in our mouse experimental model. The results of this trial confirm the absence of significant differences. A trend was noted, however, that might indicate better results with the dipropionate base in that a markedly higher therapeutic index was noted. The F value in the direct comparisons of the progressive parasitemias was 4.2 and needed to reach 5.32 to be considered significant at the 0.05 level.

The use of DMSO, alone and in combination with 4A65 has resulted in indefinite results. There is a slight indication that virulence was enhanced when DMSO was given alone, however the differences were not significant. No advantage was seen when 50% DMSO was a diluent for 4A65.

The only compound to show a marked therapeutic effect other than 4A65 was AG 74492. The activity of this material resembled 4A65 and was highly effective at a dosage of 5 mg/kg with a marked reduction in potency at the 2 and 1 mg/kg levels. Studies will be continued with this material and will include toxicity and tolerance tests. When and if an adequate supply can be obtained the effects on anaplasmosis will be measured.

The other compounds tested in these trials have failed to show therapeutic promise and will be discarded. In some instances significantly more severe reactions were detected in treated animals. The explanation for this is not clear, but at the present time it seems unwise to devote time and facilities for duplicating these results in an effort to determine if they do in fact significantly affect the course of the disease.

In summary the following compounds have proven effective:

- (1) 4A65 - 10 to 1 mg/kg (both dihydrochloride and dipropionate)

- (2) 4A65 + DMSO
- (3) AG 74492 - 40 to 5 mg/kg
- (4) AB 34313 - The activity of this compound was marginal at 20 and 40 mg/kg

Compounds tested and were ineffective as used:

- (1) Poly I/C
- (2) Peanut Oil
- (3) Freund's Complete Adjuvant
- (4) DMSO
- (5) AD 44560
- (6) AX 23187
- (7) AV 99065
- (8) AX 63047
- (9) Neoarsphenamine
- (10) 7930
- (11) Oxytetracycline (40 mg/kg level)
- (12) ZE 46212
- (13) AX 26820
- (14) AY 62009
- (15) AG 40405

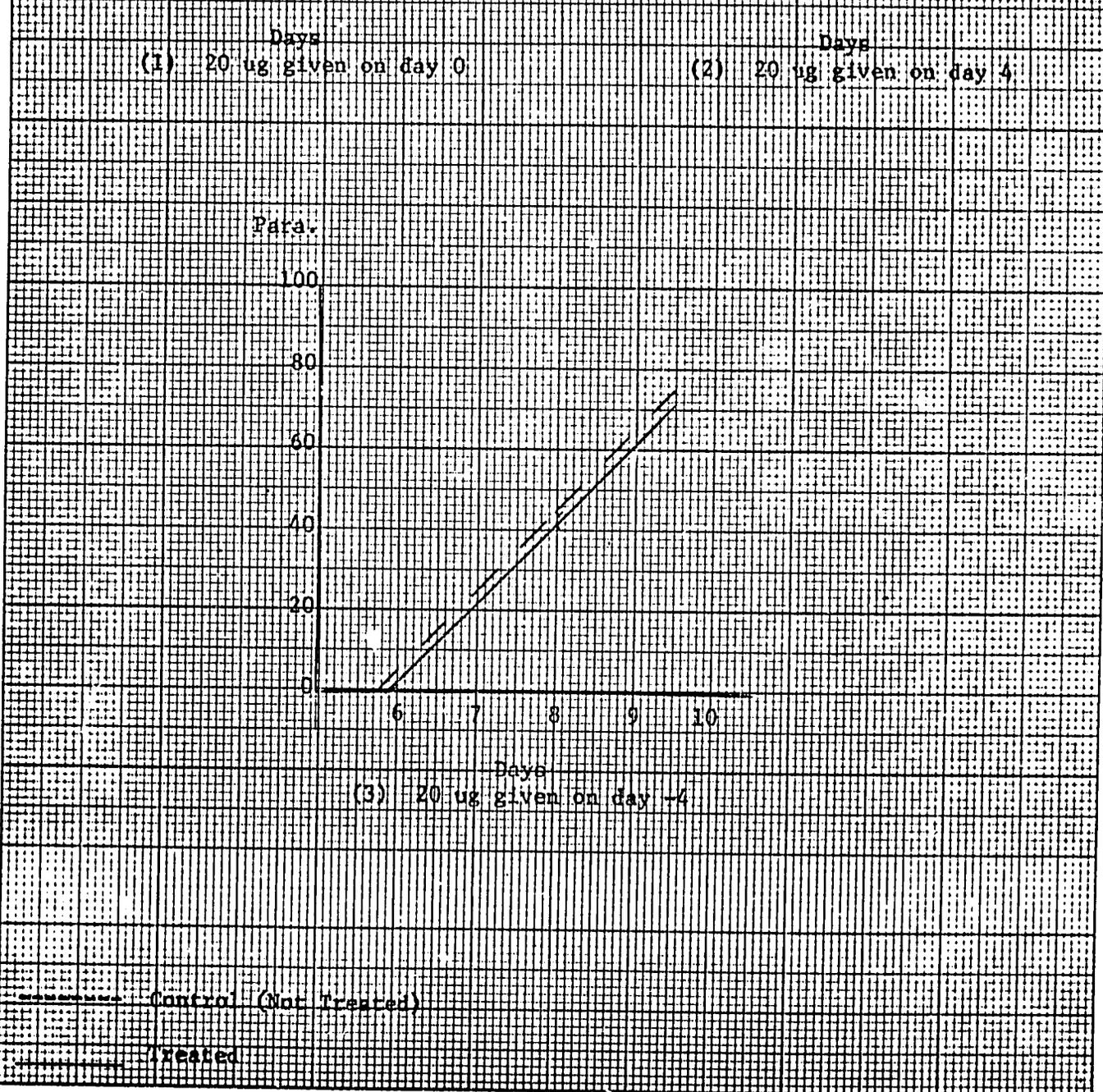
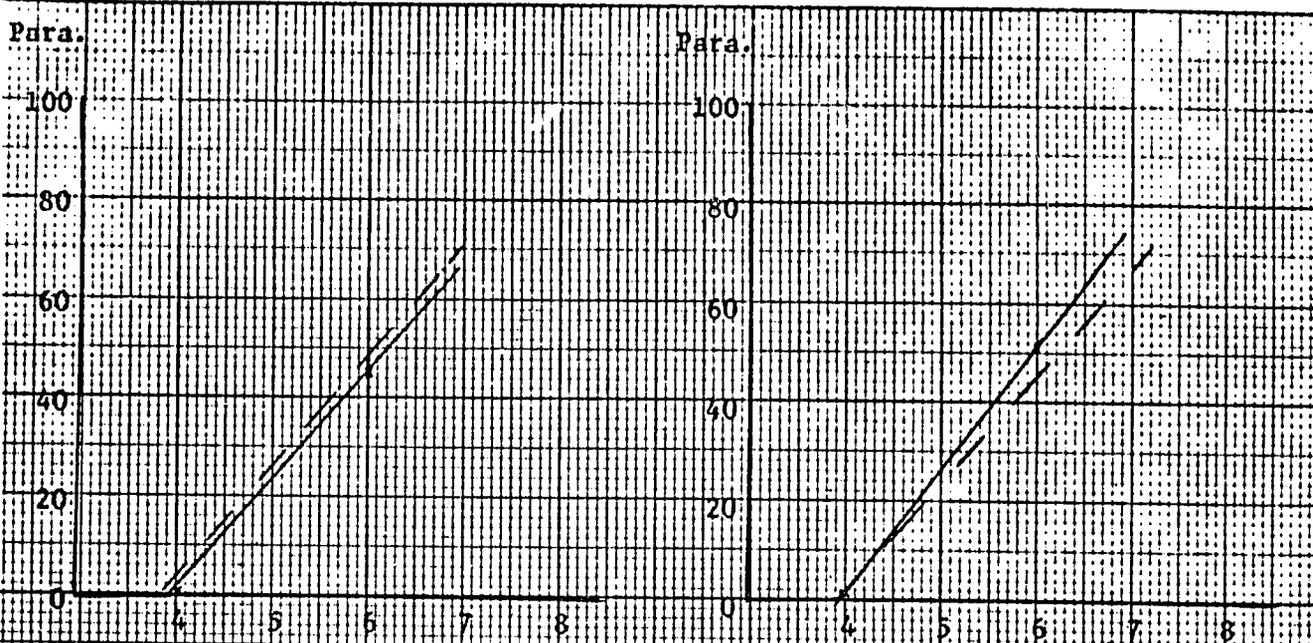
**Table 1A**  
**Drug Screening Trials Against Babesia rodhaini**

Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
Poly I/C	(i/p) 1	5.3 x 10 <sup>5</sup>	0	20.0 ug	5/5	22.6 ± 2.1	1.01	NS
	" " 2	5.3 x 10 <sup>5</sup>	4	20.0 ug	5/5	25.6 ± 0.7	0.97	NS
	" " 3	4.9 x 10 <sup>5</sup>	-4	20.0 ug	5/5	20.6 ± 4.5	1.02	NS
Freunds Complete Adjuvant (FCA)	(i/p) 4	5.3 x 10 <sup>5</sup>	0	0.1 ml	5/5	27.3 ± 1.0	0.95	NS
	" " 5	5.3 x 10 <sup>5</sup>	4	0.1 ml	5/5	24.0 ± 3.6	0.99	NS
	" " 6	4.9 x 10 <sup>5</sup>	-4	0.1 ml	5/5	14.4 ± 4.3	1.12	P<0.01
Untreated Control	7	5.3 x 10 <sup>5</sup>	-	-----	5/5	23.4 ± 4.1	----	--
	" " 8	4.9 x 10 <sup>5</sup>	-	-----	5/5	22.2 ± 1.7	----	--

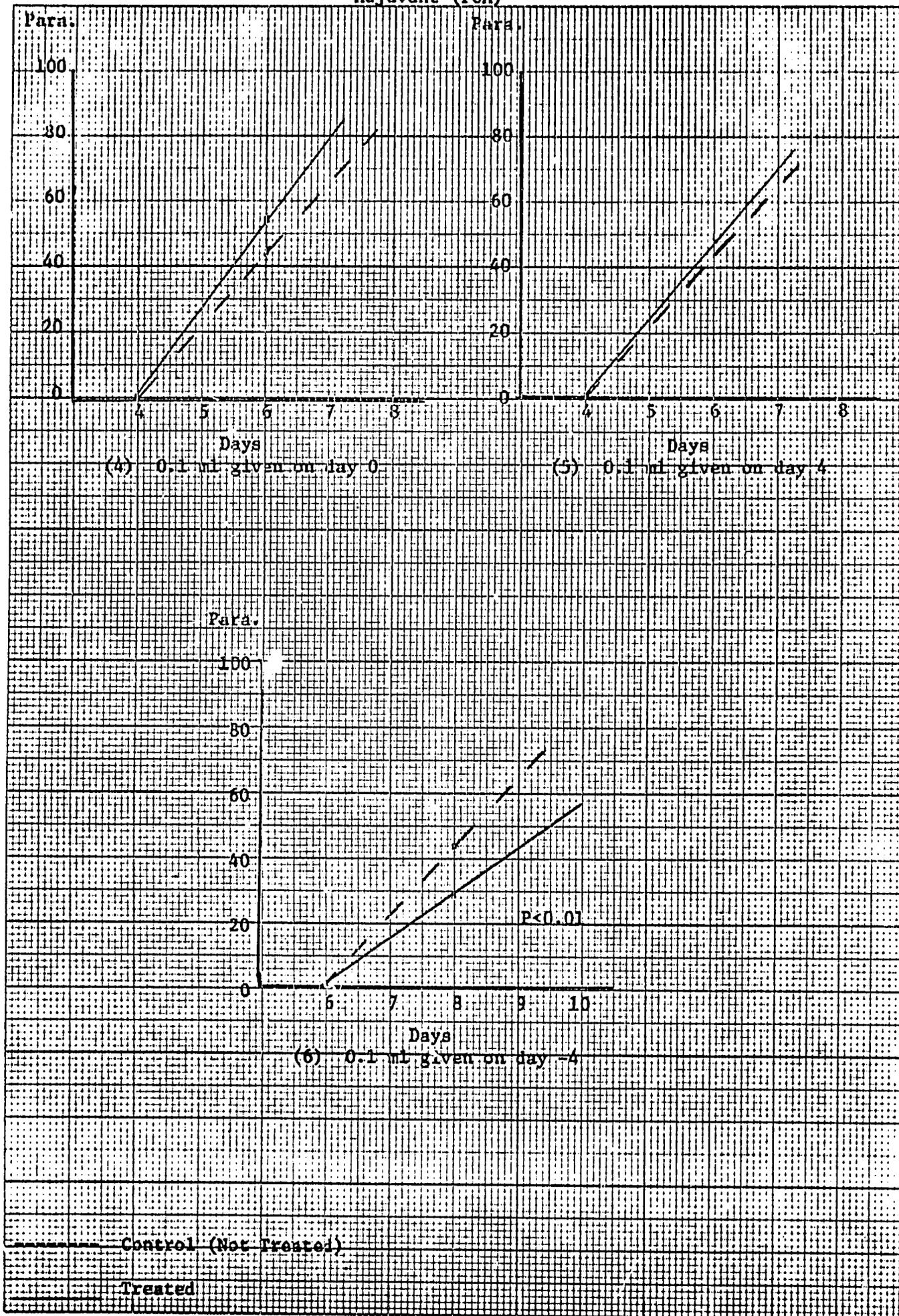
NS: Not Significant

P: Probability of Error

NO. 20 DISSEMEN UNIFORM PAPER  
20 X 20 PER INCH  
EUGENE DIFZBEN CO  
MADE IN U. S. A.



Freunds Complete Adjuvant (FCA)



EUBENE DIETZGEN CO  
MADE IN U. S. A.

10-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH

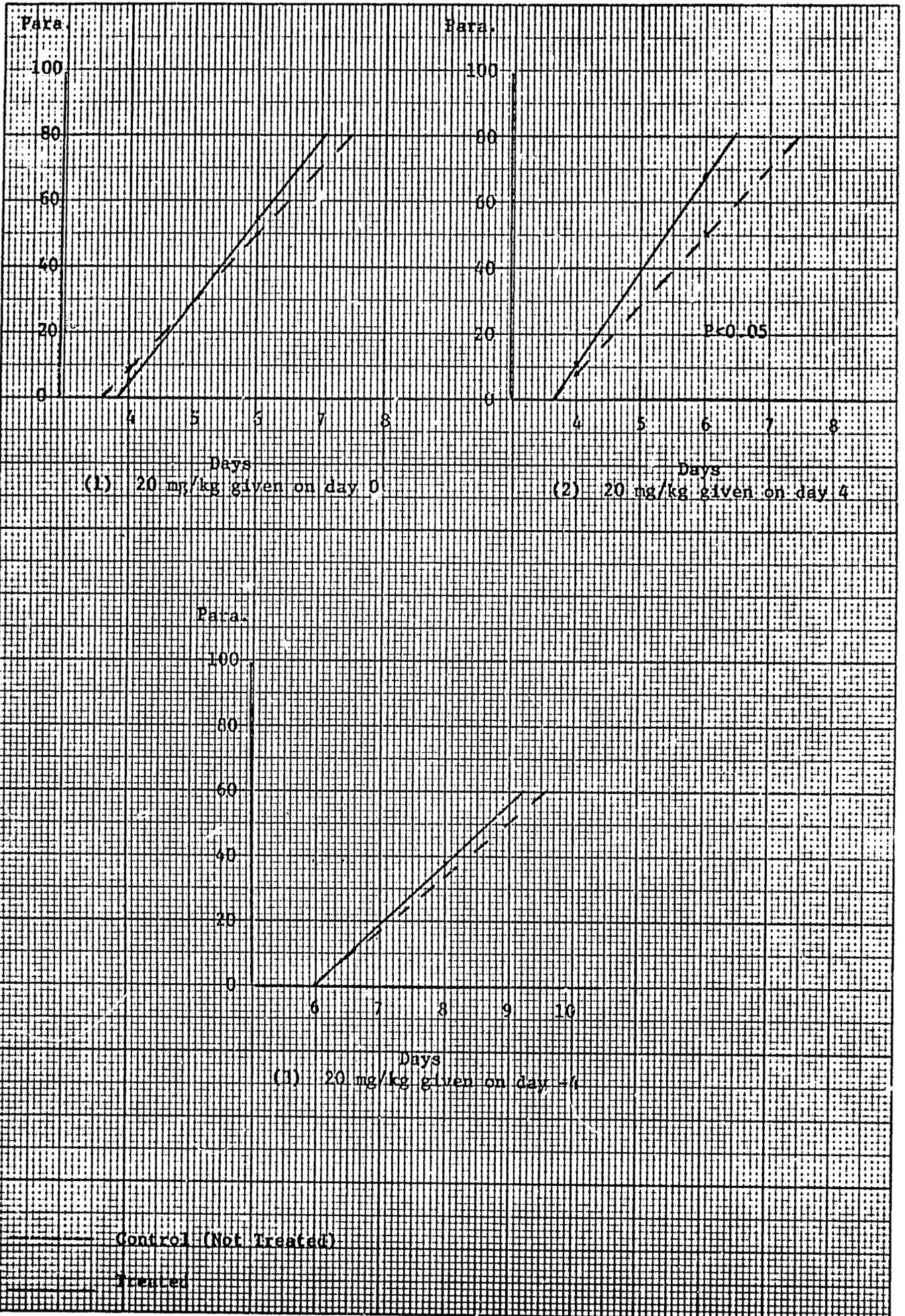
Table 1B

Drug Screening Trials Against Babesia rodhaini

Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
AD 44560	(i/p)	1	0	20 mg/kg	5/5	24.5 ± 1.6	0.95	NS
" "	"	2	4	20 mg/kg	5/5	28.8 ± 2.4	0.90	P<0.05
" "	"	3	-4	20 mg/kg	3/3	19.2 ± 0.7	0.98	NS
" "	"	4	0	40 mg/kg	3/3	25.8 ± 5.7	0.94	NS
" "	"	5	4	40 mg/kg	5/5	41.6 ± 5.8	0.77	P<0.05
" "	"	6	-4	40 mg/kg	2/2	16.8 ± 3.3	1.01	NS
Peanut Oil	(i/p)	7	0	0.6 ml	1/5	-0.6 ± 11.3	10.4	P<0.01
" "	"	8	4	0.6 ml	5/5	28.3 ± 3.2	.91	P<0.05
" "	"	9	-4	0.6 ml	4/5	15.2 ± 8.0	1.04	NS
Untreated Control		10	-	-----	5/5	20.9 ± 4.7	-----	-----
" "		11	-	-----	5/5	17.7 ± 4.0	-----	-----

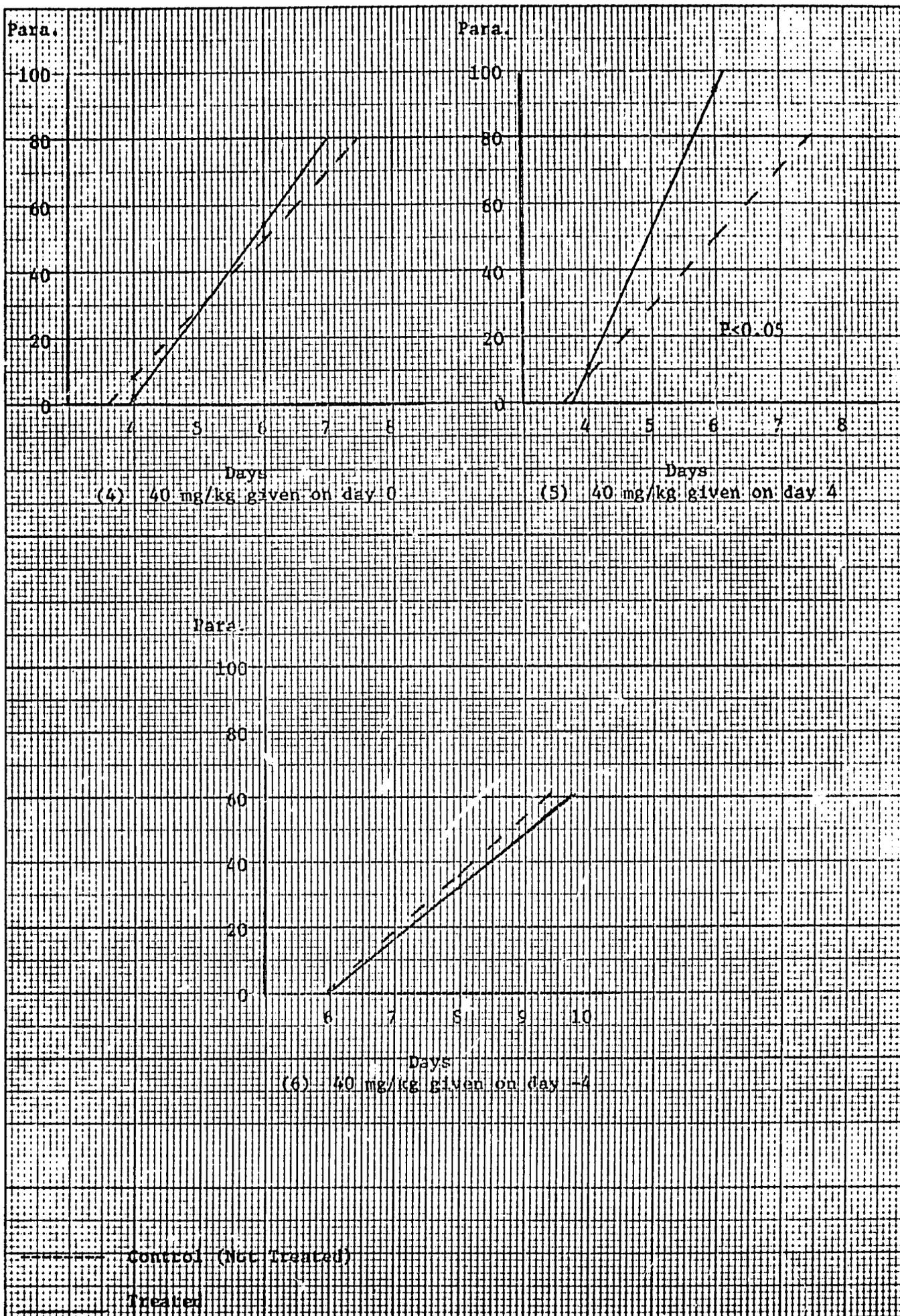
EURO-JETZ CO.  
MADE IN U. S. A.

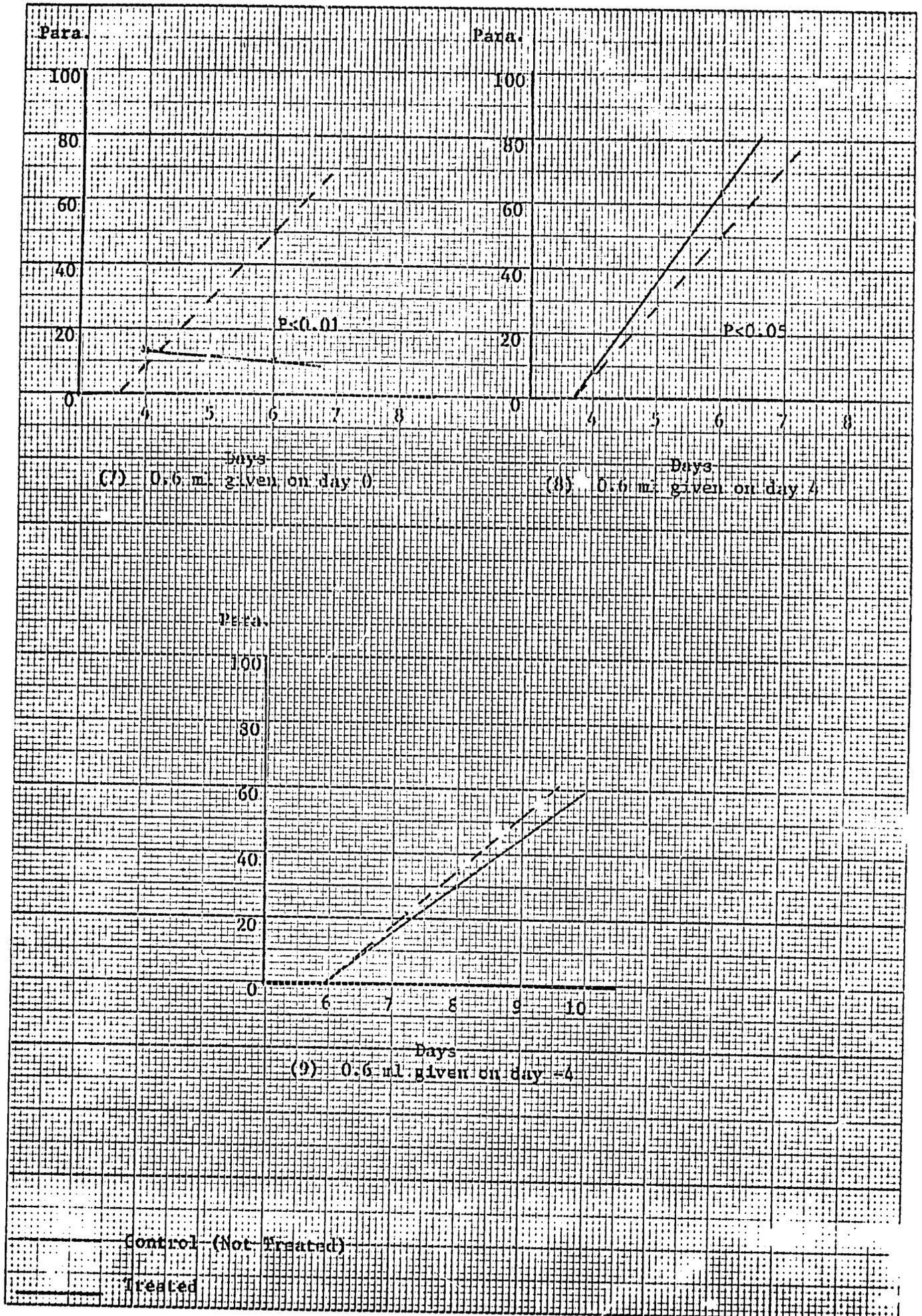
20 C. JEN PA.  
20 X 20 PER INCH



EUCLID DIETZ-CO.  
MADE IN U. S. A.

NO. 20 L. BEN H. PA  
20 X 20 PER INCH





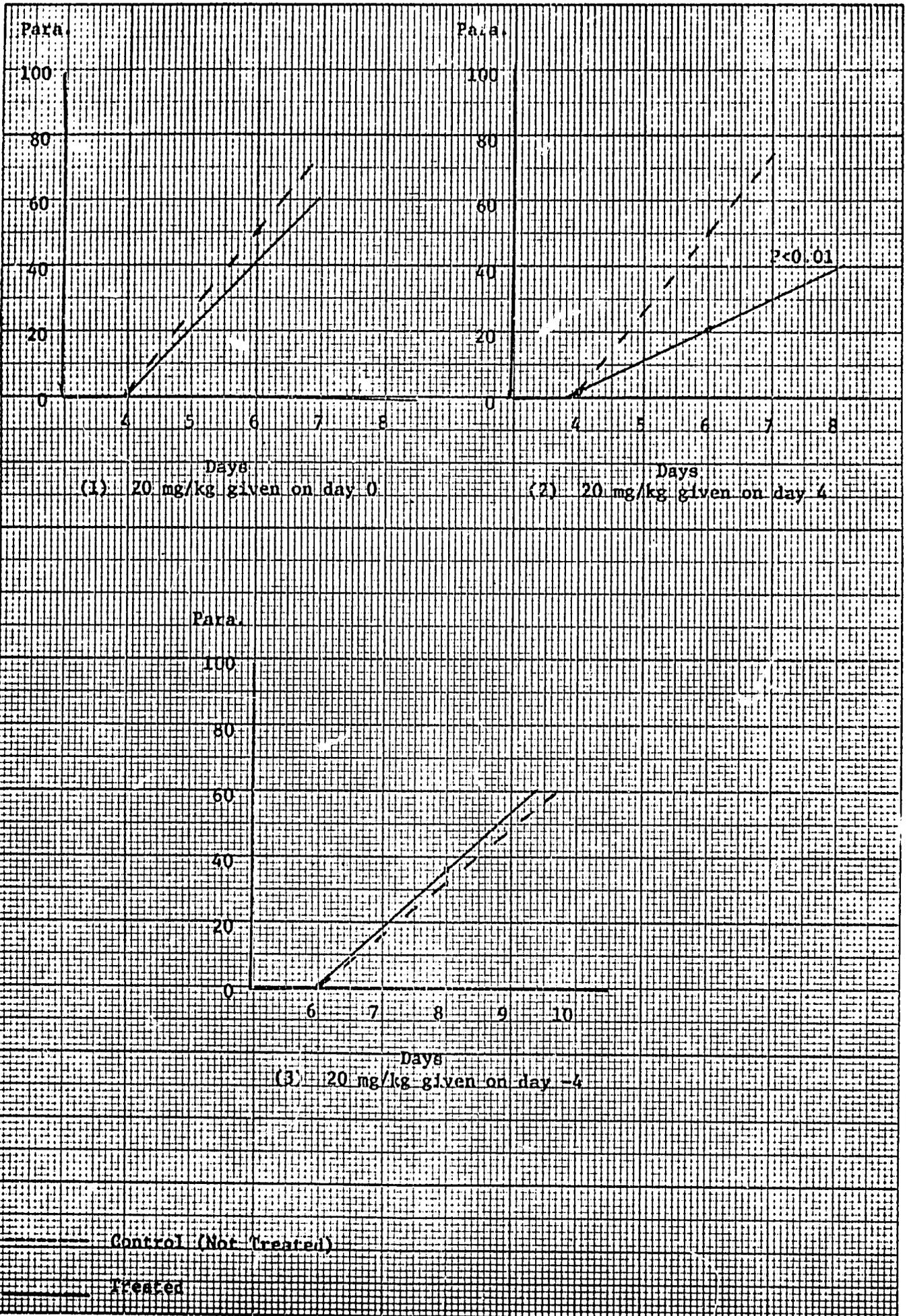
CUBENE DIETZGEN CO.  
MADE IN U. S. A.

340-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH

Table 1C

Drug Screening Trials Against Babesia rodhaini

Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
AB 34313	(s/c)	1	0	20 mg/kg	4/5	20.2 ± 10.0	1.24	NS
"	"	2	4	20 mg/kg	2/5	9.4 ± 6.0	2.54	P<0.01
"	"	3	-4	20 mg/kg	4/5	18.2 ± 5.4	0.96	NS
"	"	4	0	40 mg/kg	5/5	20.2 ± 9.8	1.07	NS
"	"	5	4	40 mg/kg	3/5	18.8 ± 14.6	1.53	NS
"	"	6	-4	40 mg/kg	5/5	19.9 ± 1.2	0.80	NS
Peanut Oil	(s/c)	8	4	0.2 ml	5/5	28.0 ± 1.2	0.96	NS
"	"	9	-4	0.2 ml	4/4	20.1 ± 1.5	0.80	NS
Untreated Control		10	-	-----	5/5	24.8 ± 3.3	----	-----
"	"	11	-	-----	5/5	16.1 ± 9.4	----	-----

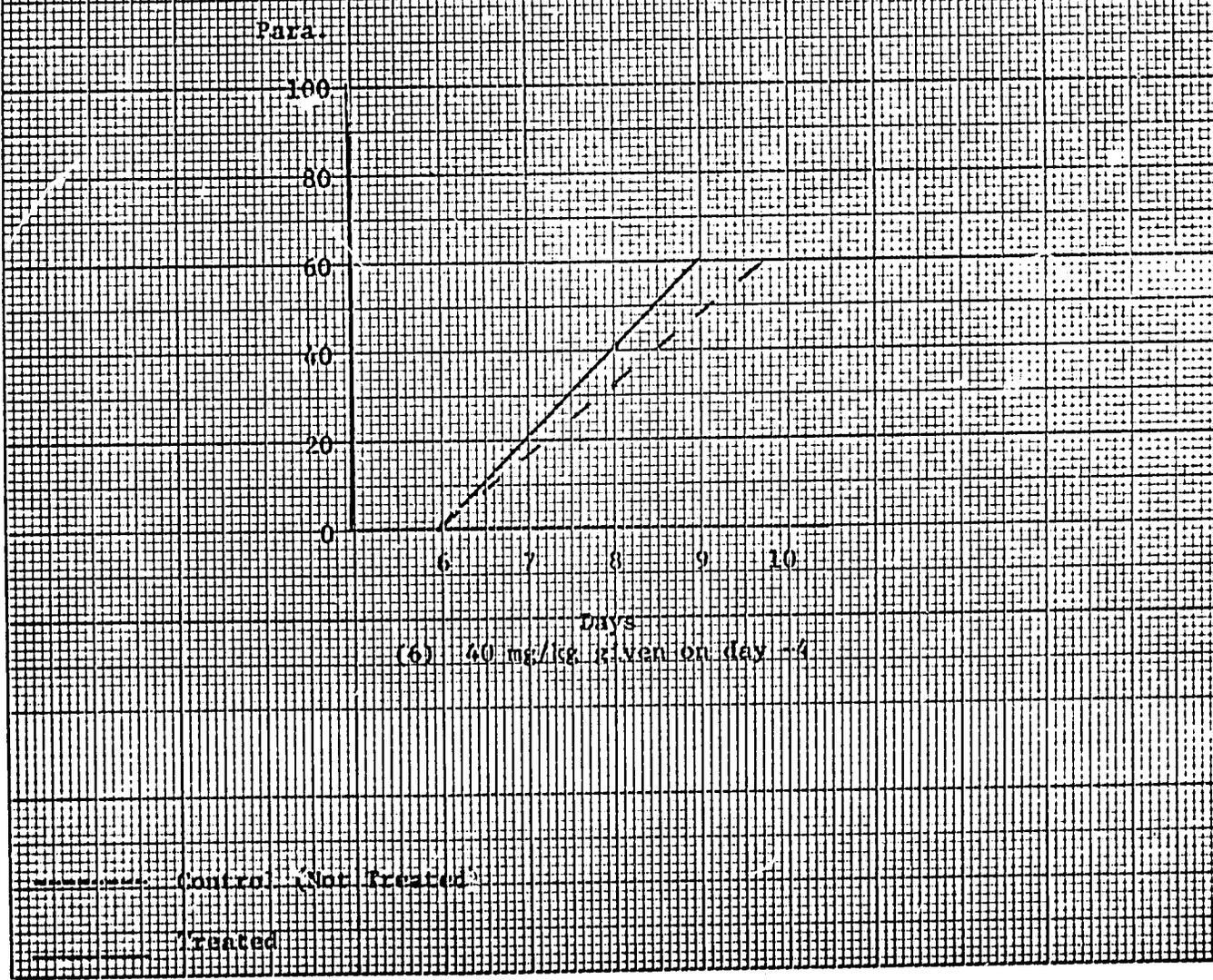
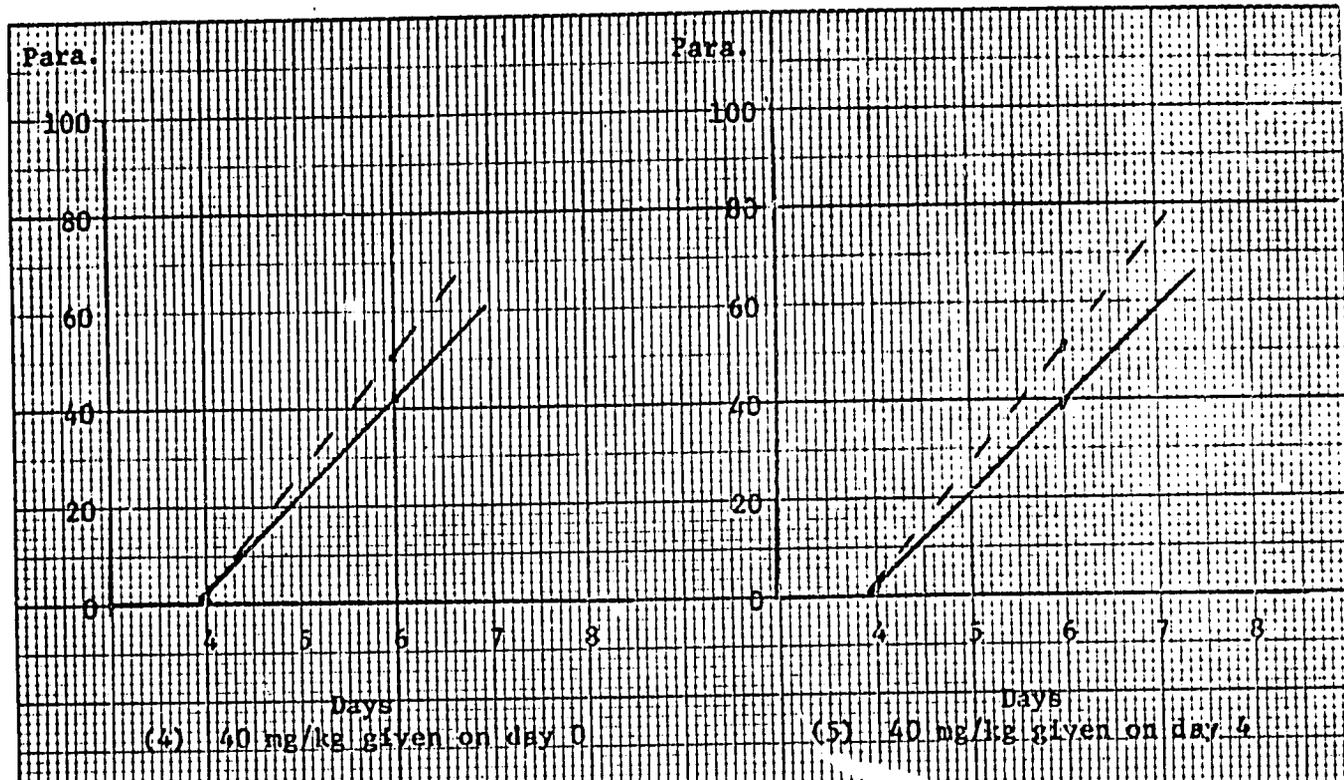


EUGENE DIETZGEN CO.  
MADE IN U. S. A.

340-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH

DIETZGEN  
EUGENE DIETZGEN CO.  
MADE IN U. S. A.

DIETZGEN  
O-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH



340-40 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH  
DIETZGEN  
LUDWIG  
KUNEN  
SUDENE DIETZGEN CO.  
MADE IN U. S. A.

Para.

100  
80  
60  
40  
20  
0

4 5 6 7 8

Days

(8) 0.2 ml given on day 4

Para.

100  
80  
60  
40  
20  
0

6 7 8 9 10

Days

(9) 0.2 ml given on day 4

Control (Not Treated)

Treated

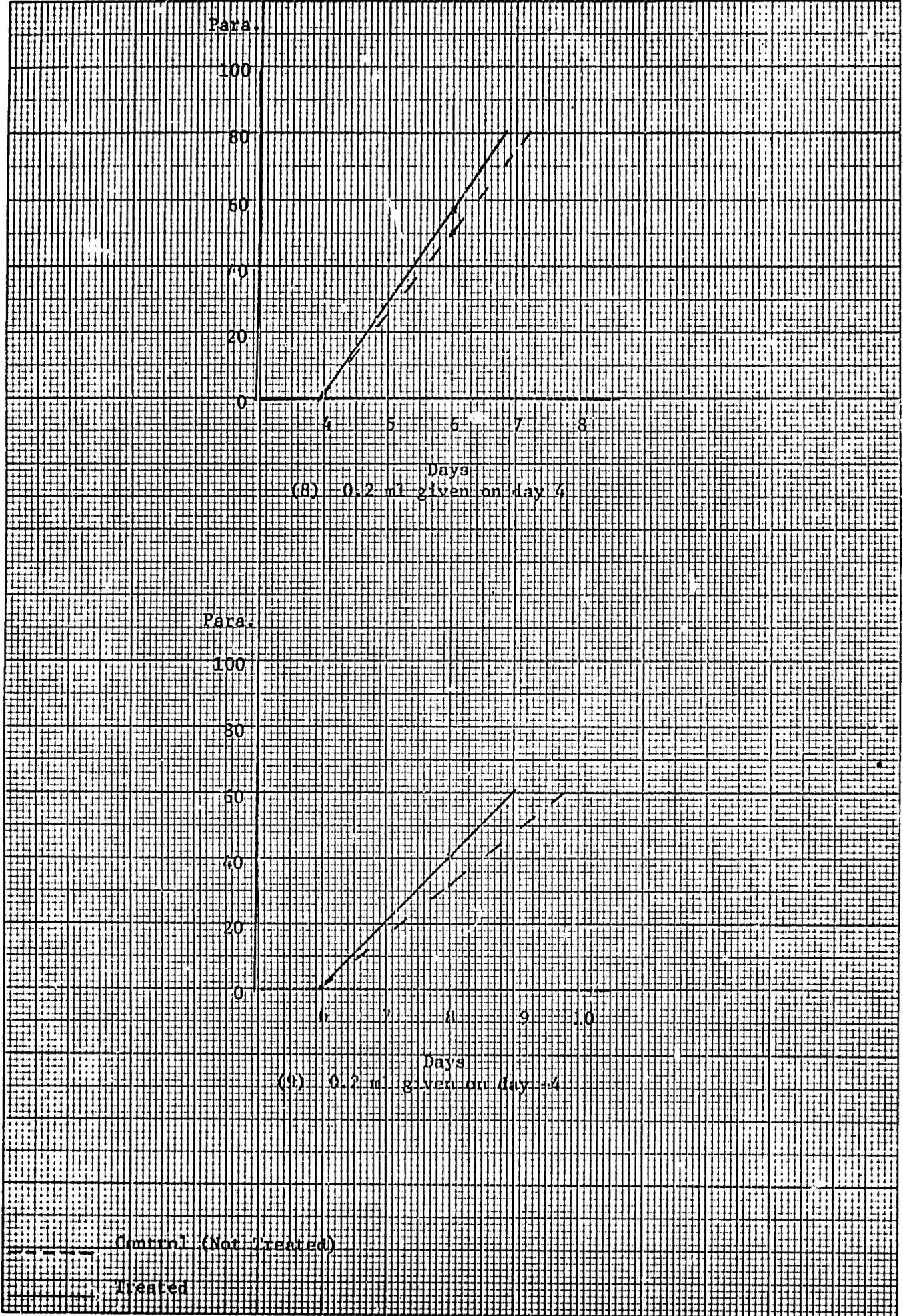
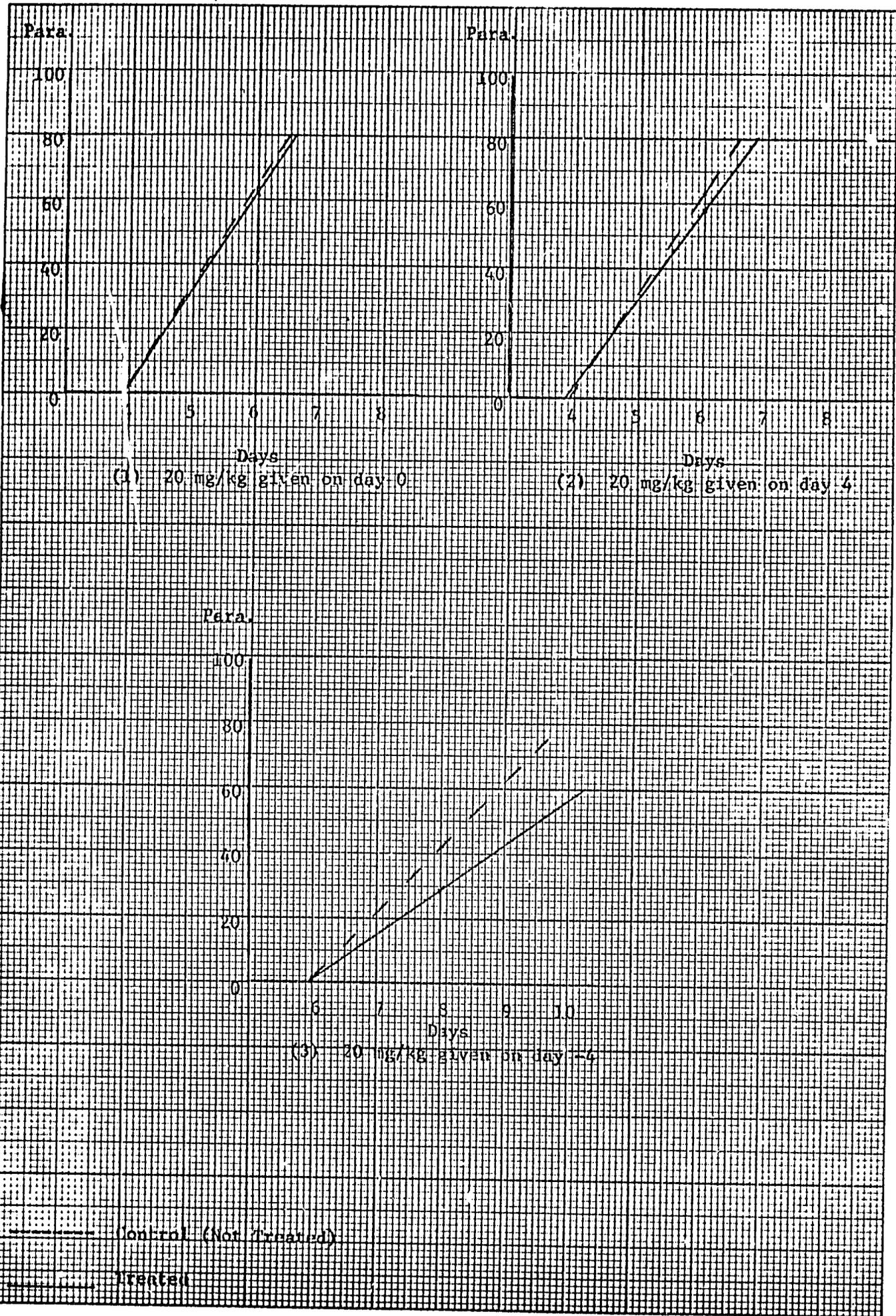


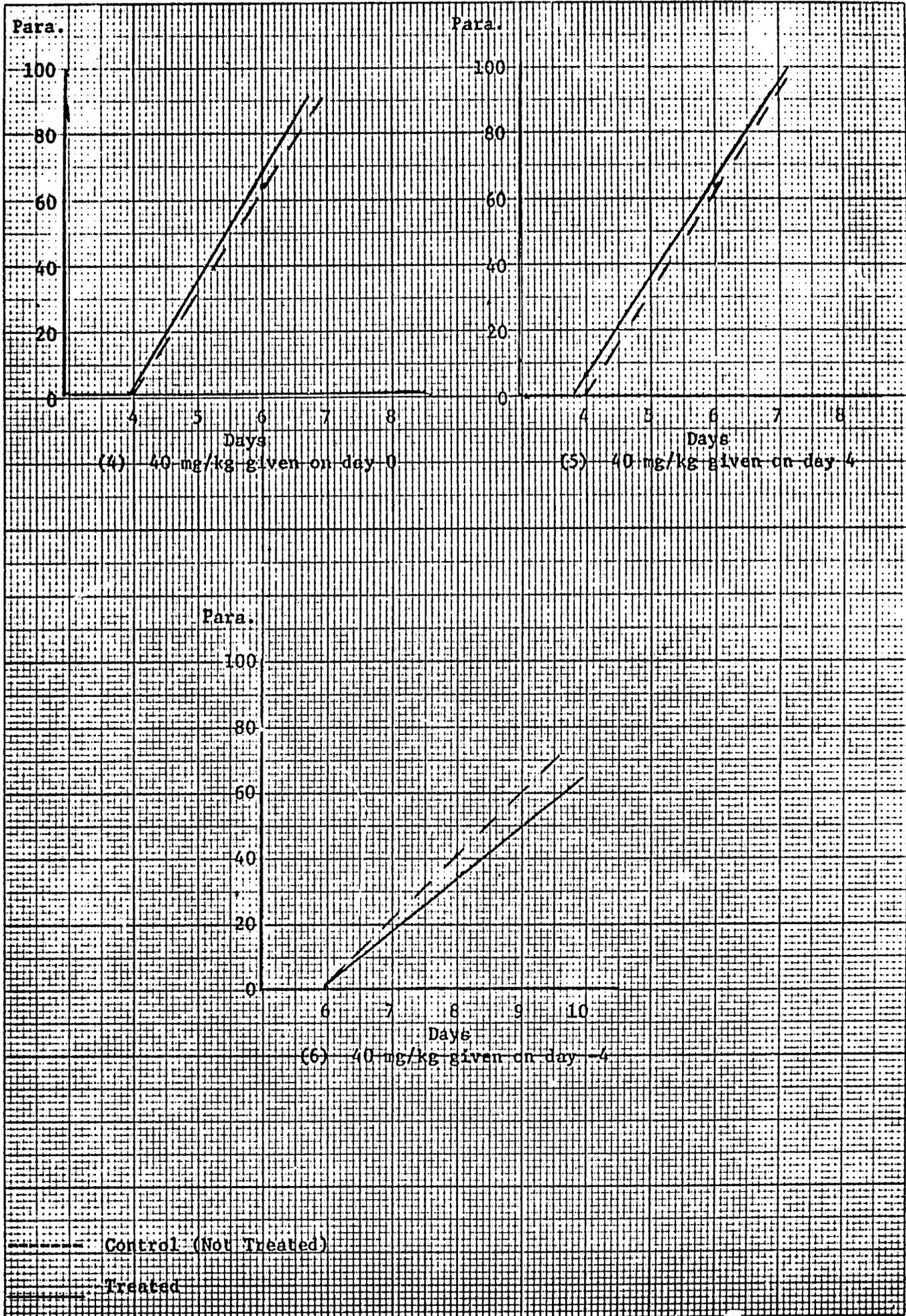
TABLE IV  
Drug Screening Trials Against Babesia rodhaini

Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
AX 23187	(s/c)	1	0	20 mg/kg	5/5	29.6 ± 1.5	1.01	NS
" "	"	2	4	20 mg/kg	5/5	27.2 ± 2.8	1.04	NS
" "	"	3	-4	20 mg/kg	4/5	14.6 ± 7.0	1.30	NS
" "	"	4	0	40 mg/kg	5/5	32.1 ± 1.7	0.98	NS
" "	"	5	4	40 mg/kg	5/5	29.7 ± 4.3	1.01	NS
" "	"	6	-4	40 mg/kg	4/5	17.7 ± 7.4	1.05	NS
Peanut Oil	(s/c)	7	0	0.1 ml	5/5	27.1 ± 1.6	1.04	NS
" "	"	8	4	0.1 ml	5/5	31.0 ± 2.2	0.99	NS
" "	"	9	-4	0.1 ml	5/5	19.8 ± 4.5	1.02	NS
Untreated Control		10	-	-----	5/5	30.4 ± 4.2	----	--
" "	"	11	-	-----	5/5	20.9 ± 1.8	----	--

EUGENE DIETZGEN CO.  
MADE IN U. S. A.

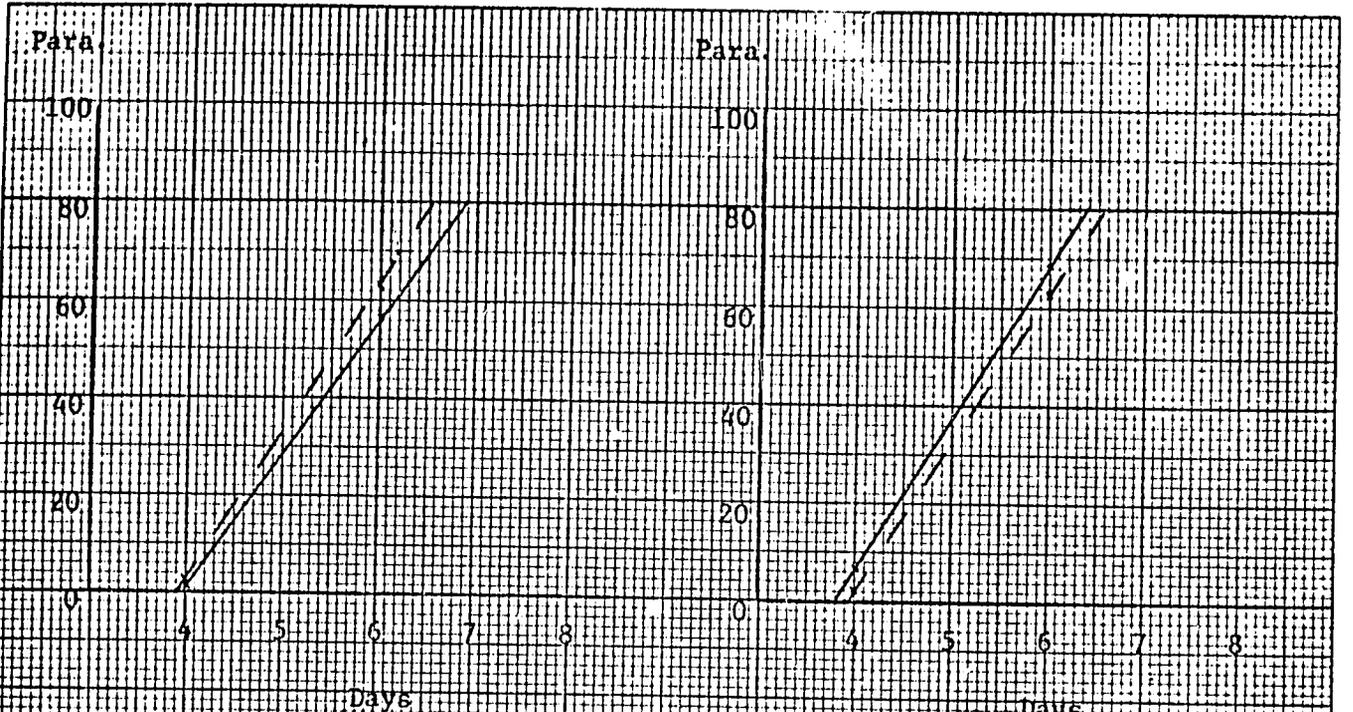
34-J-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH





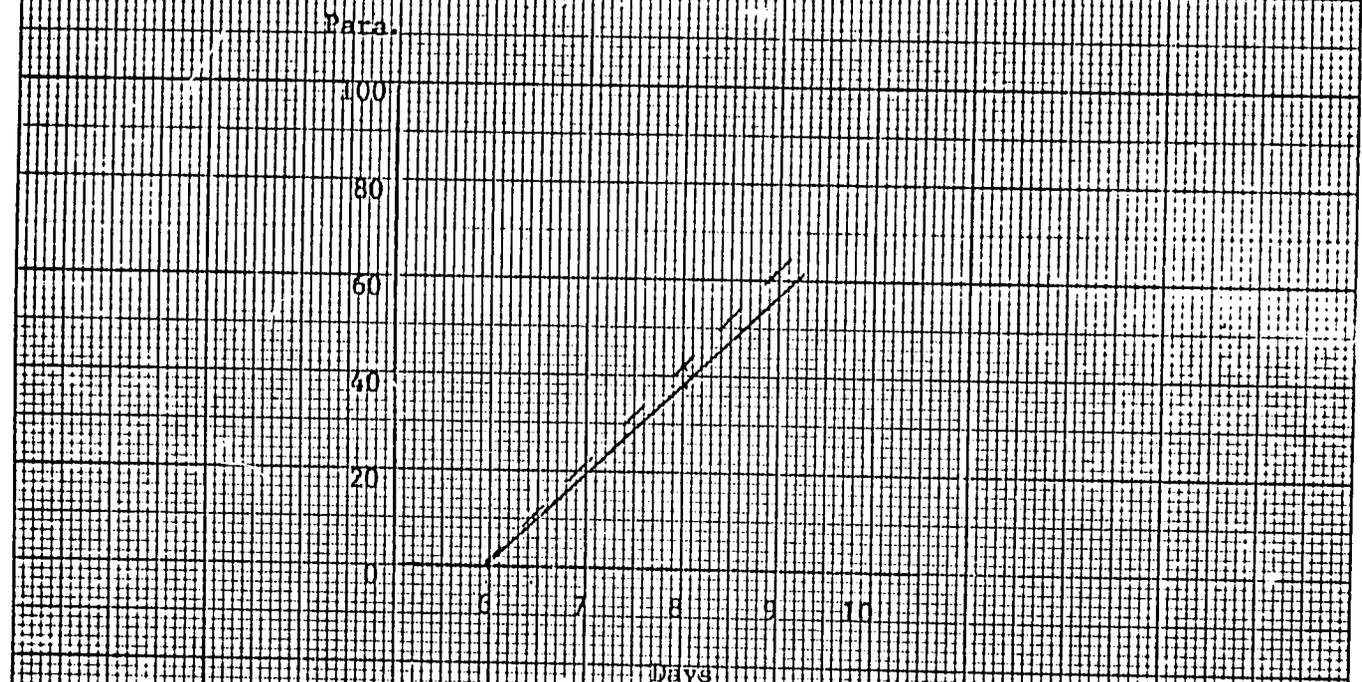
EUGENE DIETZGEN CO.  
MADE IN U. S. A.

502U DIETZGEN GRAPH PAPER  
20 X 20 PER INCH



(7) 0.1 ml peanut oil given on day 0

(8) 0.1 ml peanut oil given on day 4



(9) 0.1 ml peanut oil given on day -7

Control (Not Treated)

Treated

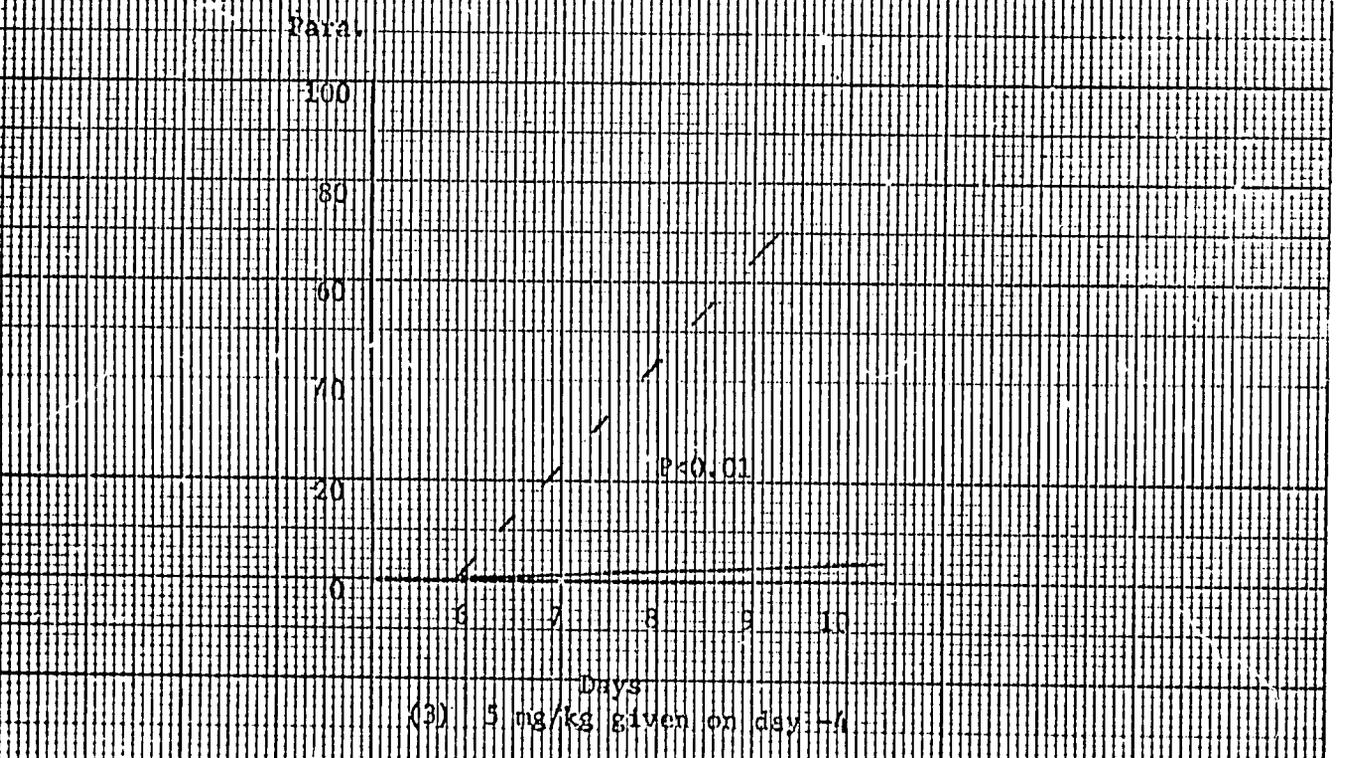
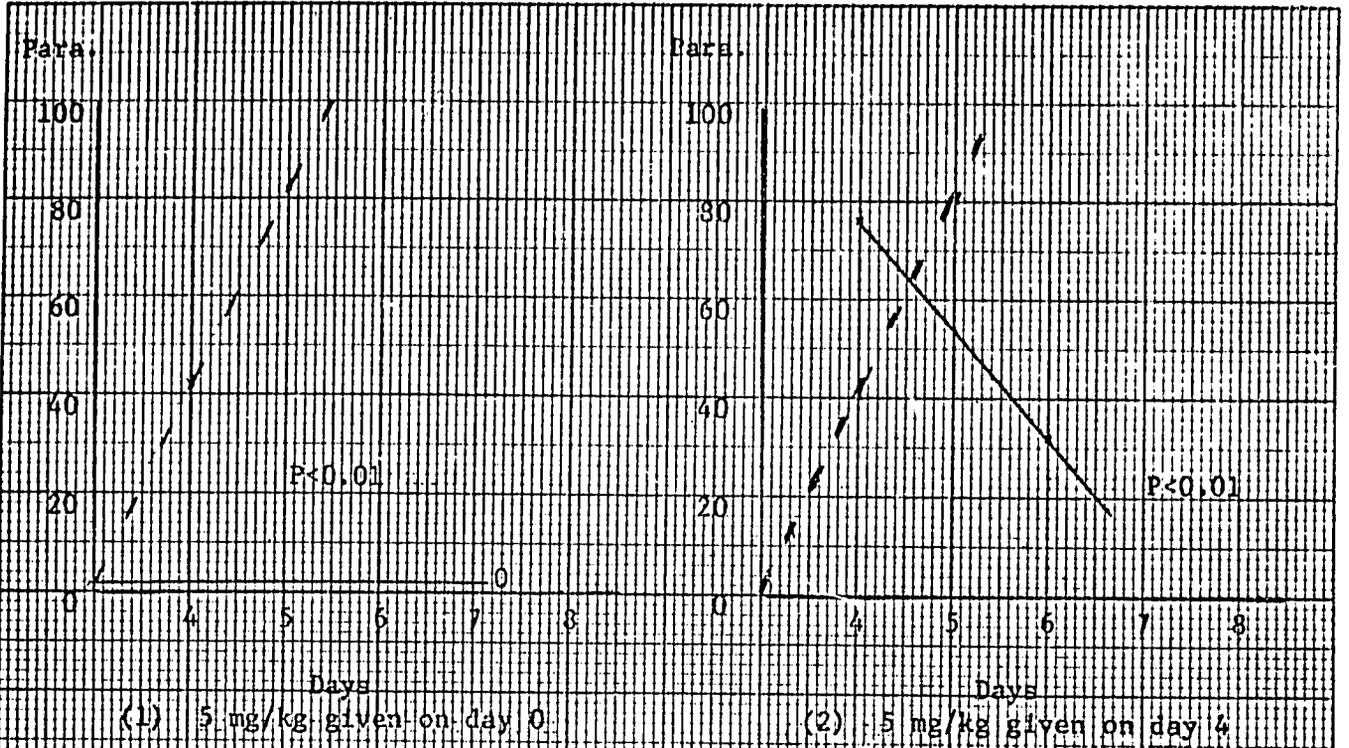
DUPLICATE COPY  
MADE IN U. S. A.

NO. 3-4-44 DIETETIC GRAM PAPER  
20 X 20 PER INCH

Table 1E

Drug Screening Trials Against Babesia rodhaini

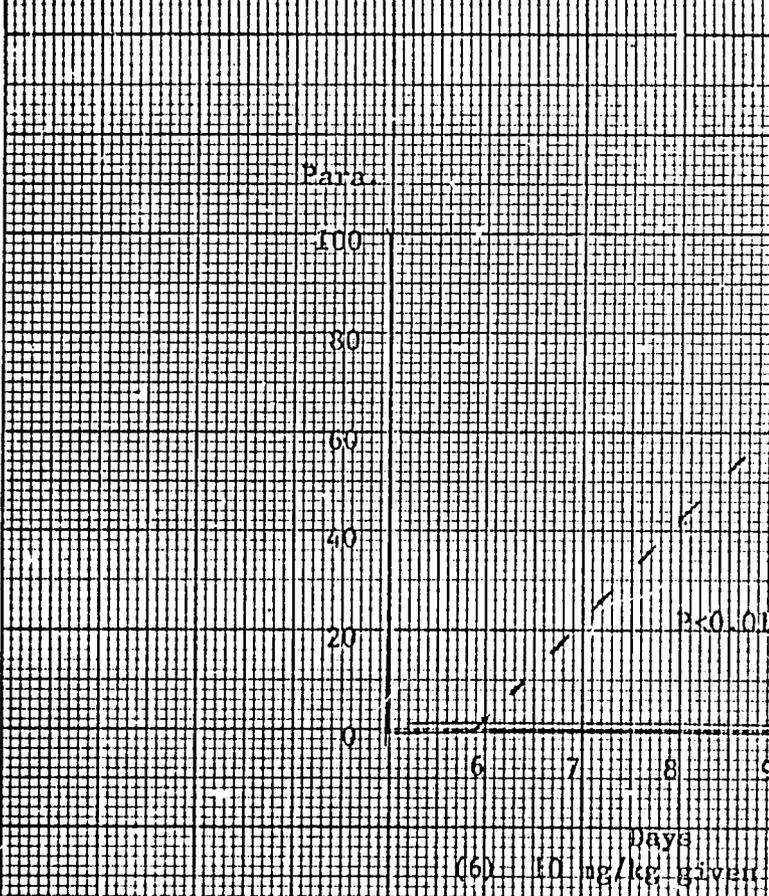
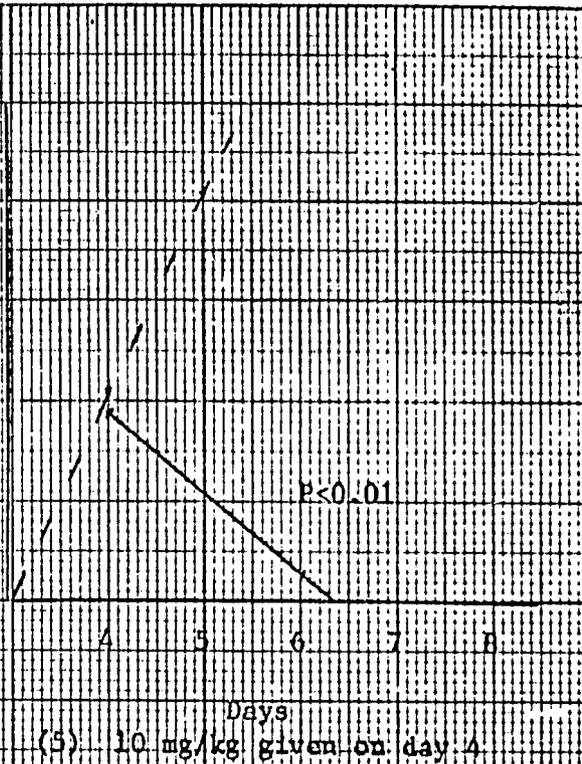
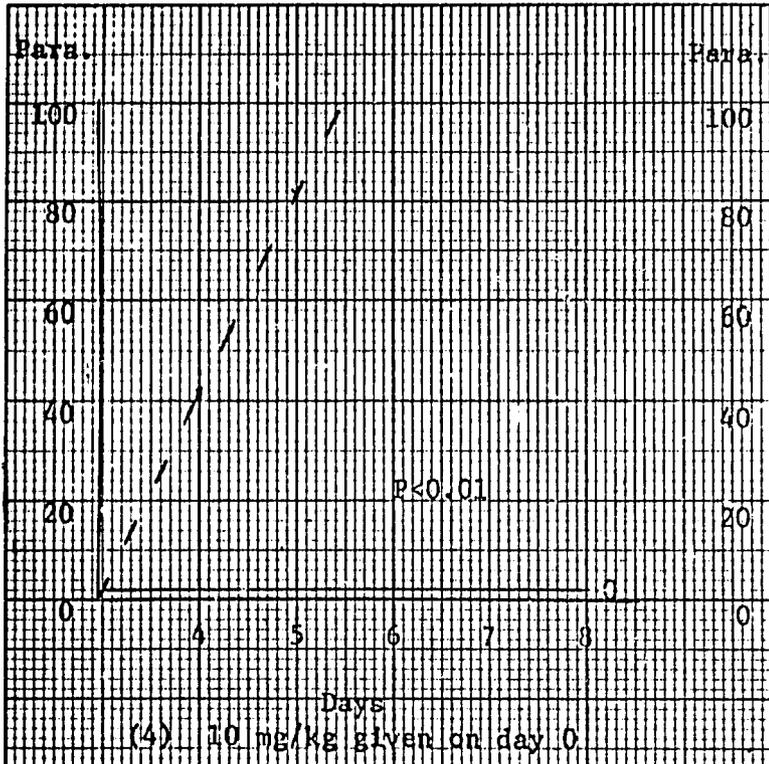
Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
4A65	(s/c) 1	$5.7 \times 10^5$	0	5 mg/kg	0/4	0	>90.2	P<0.01
"	" 2	$5.7 \times 10^5$	4	5 mg/kg	1/5	-22.4 ± 9.7	9.0	P<0.01
"	" 3	$5.9 \times 10^5$	-4	5 mg/kg	0/5	0.9 ± 1.7	76.0	P<0.01
"	" 4	$5.7 \times 10^5$	0	10 mg/kg	0/5	0	>90.2	P<0.01
"	" 5	$5.7 \times 10^5$	4	10 mg/kg	0/5	-15.9 ± 2.5	>90.2	P<0.01
"	" 6	$5.9 \times 10^5$	-4	10 mg/kg	0/5	0	>70.2	P<0.01
Untreated Control	7	$5.7 \times 10^5$	-	-----	5/5	40.2 ± 15.9	-----	-----
"	8	$5.9 \times 10^5$	-	-----	5/5	20.3 ± 0.9	-----	-----



----- Control (Not Treated)  
 \_\_\_\_\_ Treated

LUBENE WETZON CO.  
 MADE IN U. S. A.

ml. 347-200 DIET AND GRASS PAPER  
 20 X 20 PER INCH



----- Control (Not Treated)  
 - - - - - Treated

20 X 20 PER INCH  
 EUGENE JETZEN CO.  
 MADE IN U. S. A.

Table 1F

Drug Screening Trials Against Babesia rodhaini

Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
AV 99065	(s/c)	1	0	20 mg/kg	5/5	31.5 ± 5.0	0.82	NS
"	"	2	4	20 mg/kg	5/5	29.7 ± 2.7	0.84	NS
"	"	4	0	40 mg/kg	5/5	31.1 ± 3.3	0.82	NS
"	"	5	4	40 mg/kg	5/5	30.9 ± 3.0	0.82	NS
"	"	6	-4	40 mg/kg	5/5	17.9 ± 4.6	1.01	NS
Untreated Control		7	-	-----	4/5	26.7 ± 4.4	----	--
"	"	8	-	-----	5/5	18.9 ± 2.4	----	--

EUSCENE DIETZGEN CO.  
MADE IN U. S. A.

NO. 340-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH

Para.

100

80

60

40

20

0

4

5

6

7

8

Days

(1) 20 mg/kg given on day 0

Para.

100

80

60

40

20

0

4

5

6

7

8

Days

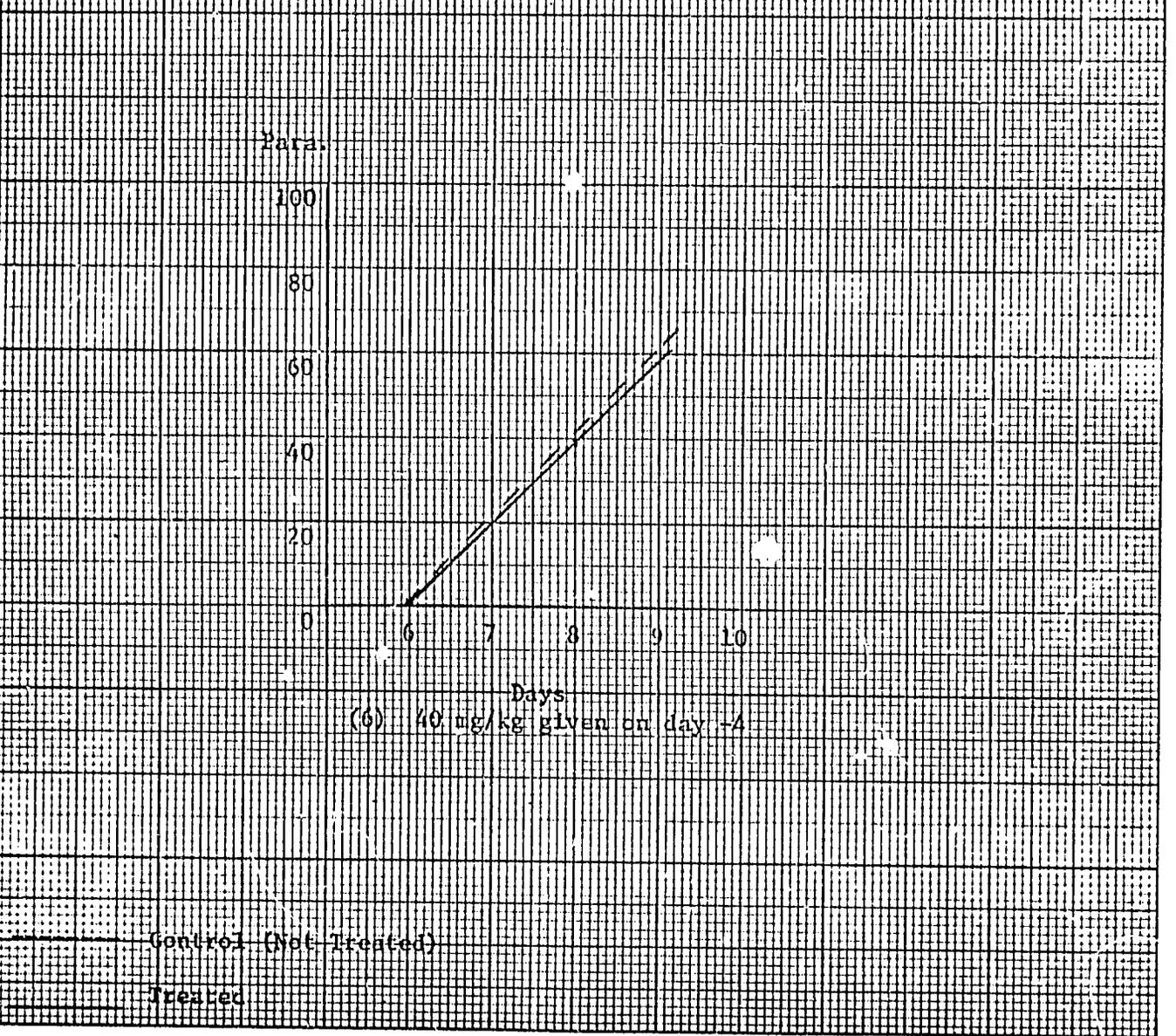
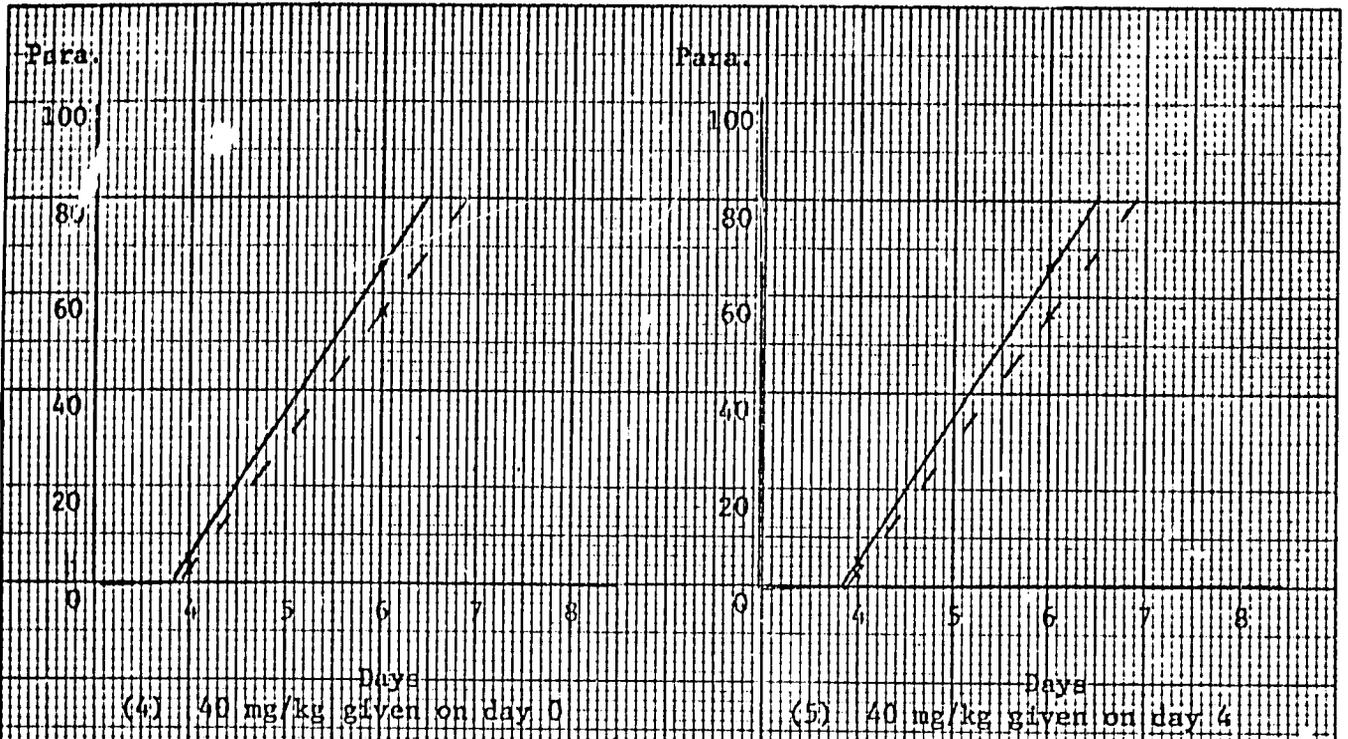
(2) 20 mg/kg given on day 4

Control (Not Treated)

Treated

ALBEMAR CHEMICAL CO.  
MADE IN U. S. A.

340-100 JIETZ... GRAF... PER  
20 X 20 PER INCH



Control (Not Treated)  
Treated

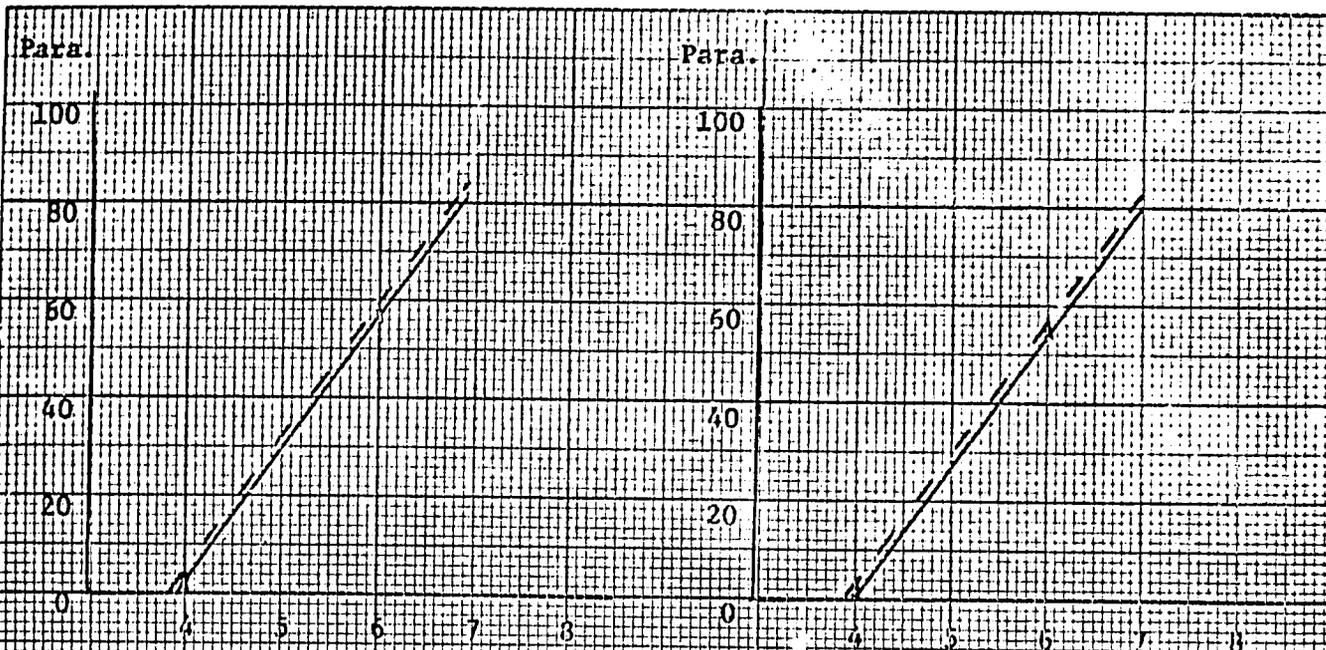
Table 1G

Drug Screening Trials Against Babesia rodhaini

Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
AX 63047	(s/c) 1	8.6 x 10 <sup>5</sup>	0	20 mg/kg	4/5	26.5 ± 13.3	1.00	NS
" "	" 2	8.6 x 10 <sup>5</sup>	4	20 mg/kg	4/5	26.3 ± 9.2	1.00	NS
" "	" 3	9.6 x 10 <sup>5</sup>	-4	20 mg/kg	4/5	17.3 ± 5.3	1.06	NS
" "	" 4	8.6 x 10 <sup>5</sup>	0	40 mg/kg	5/5	28.3 ± 3.4	0.98	NS
" "	" 5	8.6 x 10 <sup>5</sup>	4	40 mg/kg	5/5	26.7 ± 3.2	1.00	NS
" "	" 6	9.6 x 10 <sup>5</sup>	-4	40 mg/kg	3/5	18.5 ± 3.5	1.04	NS
Untreated Control	7	8.6 x 10 <sup>5</sup>	-	-----	5/5	26.5 ± 8.7	----	--
" "	8	9.6 x 10 <sup>5</sup>	-	-----	5/5	21.1 ± 0.8	----	--

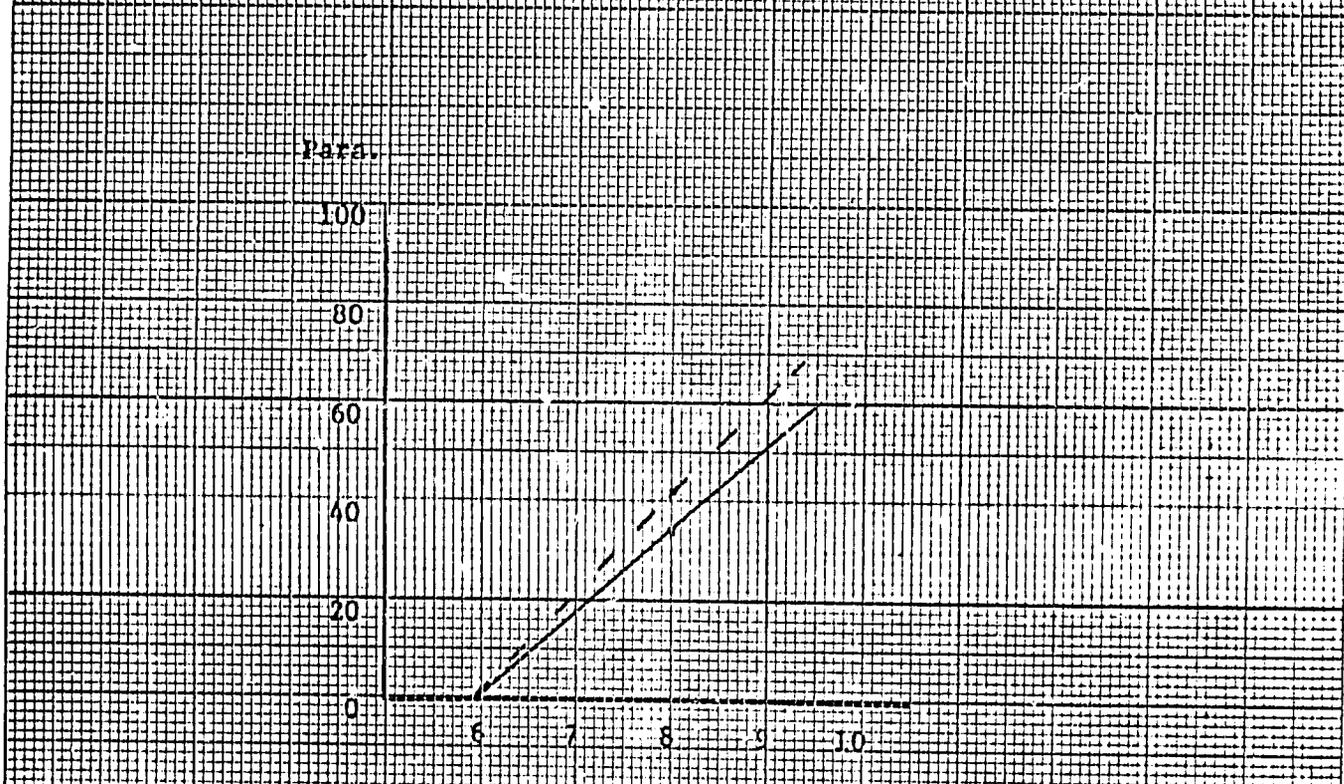
EUGENE DIETZGEN CO.  
MADE IN U. S. A.

NO. 340-20 DIETZGEN GRAPH PAPER  
20 X 20 PER 11.25M



(1) 20 mg/kg given on day 0

(2) 20 mg/kg given on day 4



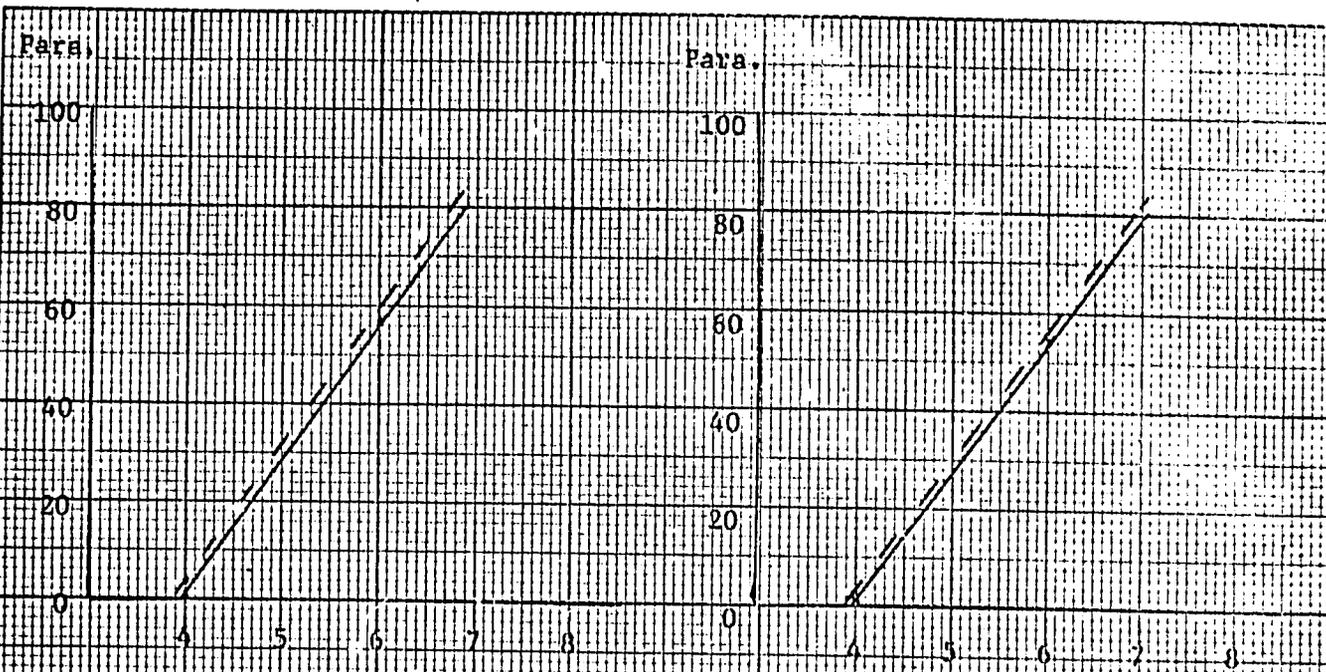
(3) 20 mg/kg given on day -3

Control (Not Treated)

Treated

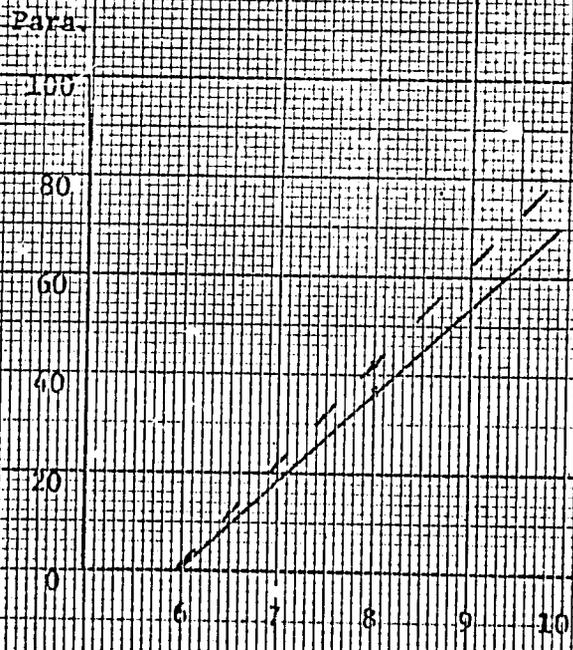
EUGENE DIETZGEN CO.  
MADE IN U. S. A.

NO. 340-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH



Days  
(4) 40 mg/kg given on day 0

Days  
(5) 40 mg/kg given on day 4



Days  
(6) 40 mg/kg given on day -4

----- Control (Not Treated)

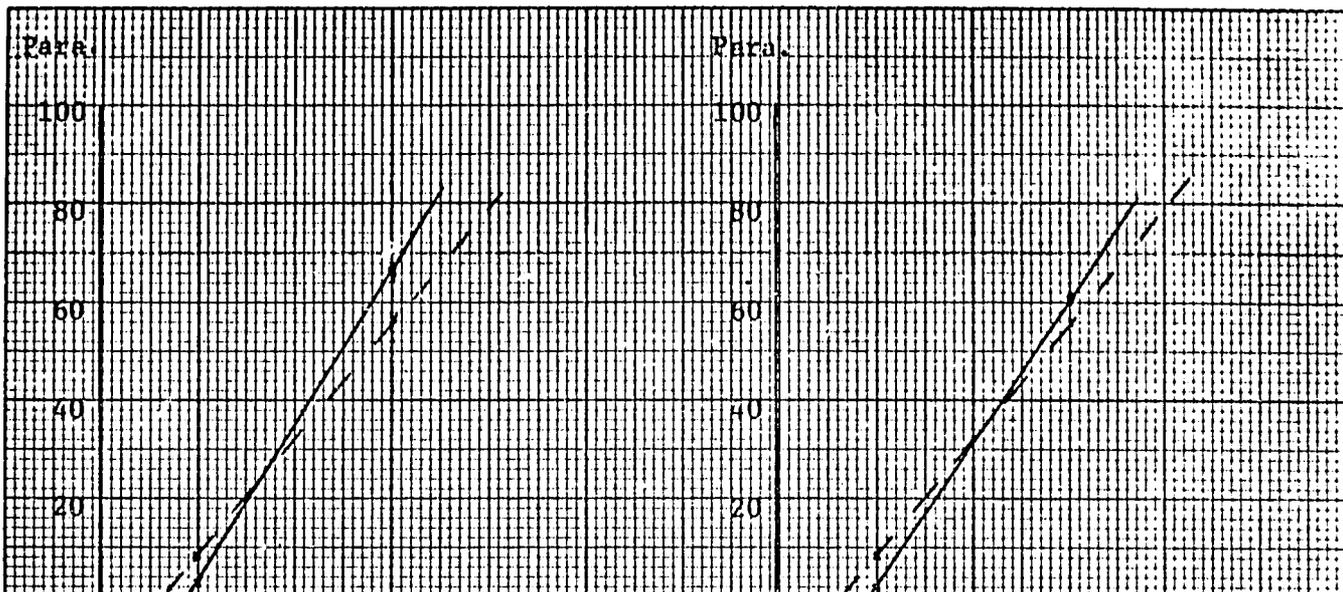
----- Treated

**Table 1H**  
**Drug Screening Trials Against Babesia rodhaini**

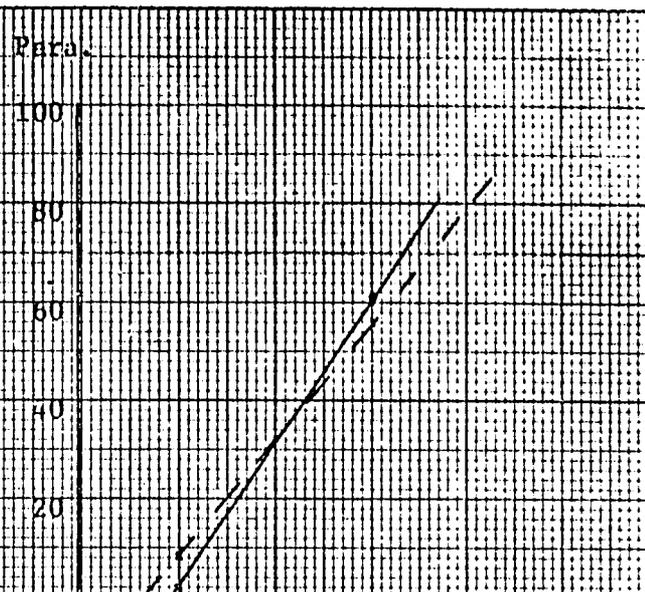
Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
AD 44560	(s/c)	1	0	20 mg/kg	5/5	31.2 ± 10.0	0.92	NS
"	"	2	4	20 mg/kg	4/5	29.1 ± 15.2	0.94	NS
"	"	3	-4	20 mg/kg	4/5	18.6 ± 3.2	1.03	NS
"	"	4	0	40 mg/kg	4/5	29.7 ± 12.2	0.93	NS
"	"	5	4	40 mg/kg	5/5	29.9 ± 6.8	0.93	NS
"	"	6	-4	40 mg/kg	5/5	19.4 ± 2.3	1.01	NS
Untreated Control		7	-	-----	5/5	24.4 ± 6.0	----	--
"	"	8	-	-----	4/5	20.1 ± 3.4	----	--

EUGENE DIETZGEN CO.  
MADE IN U. S. A.

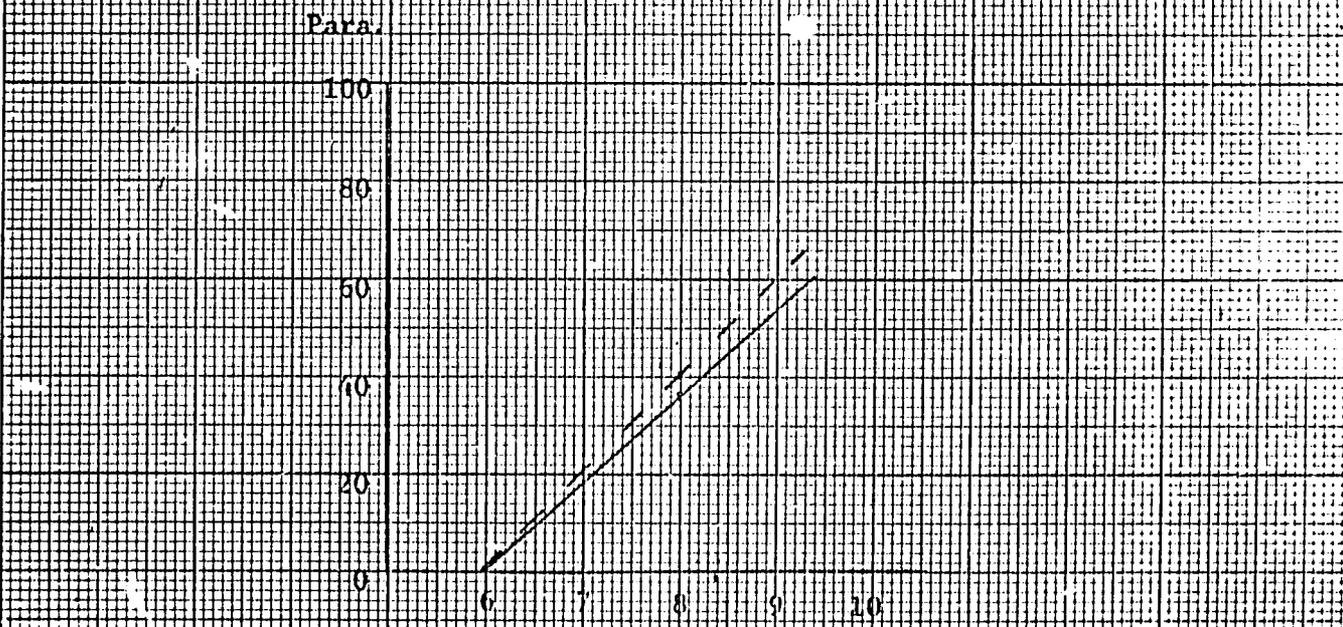
NO. 340-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH



(1) 20 mg/kg given on day 0



(2) 20 mg/kg given on day 4



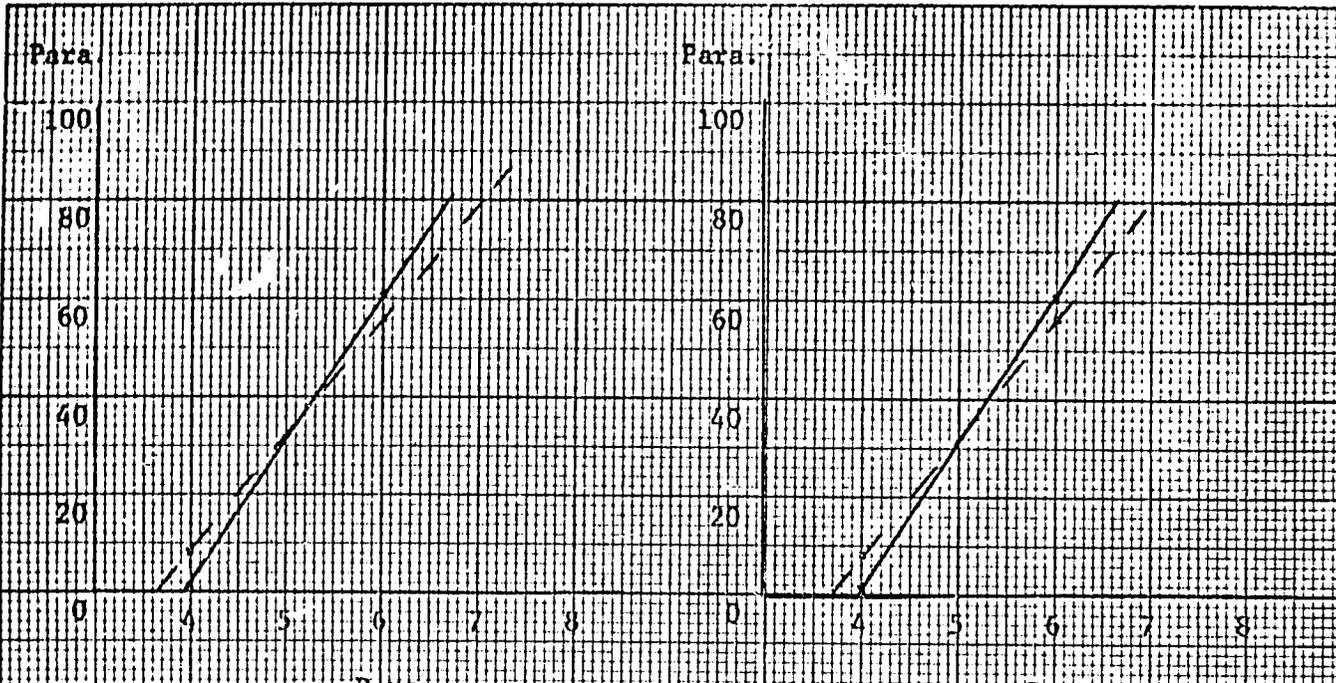
(3) 20 mg/kg given on day -4

Control (Not Treated)

Treated

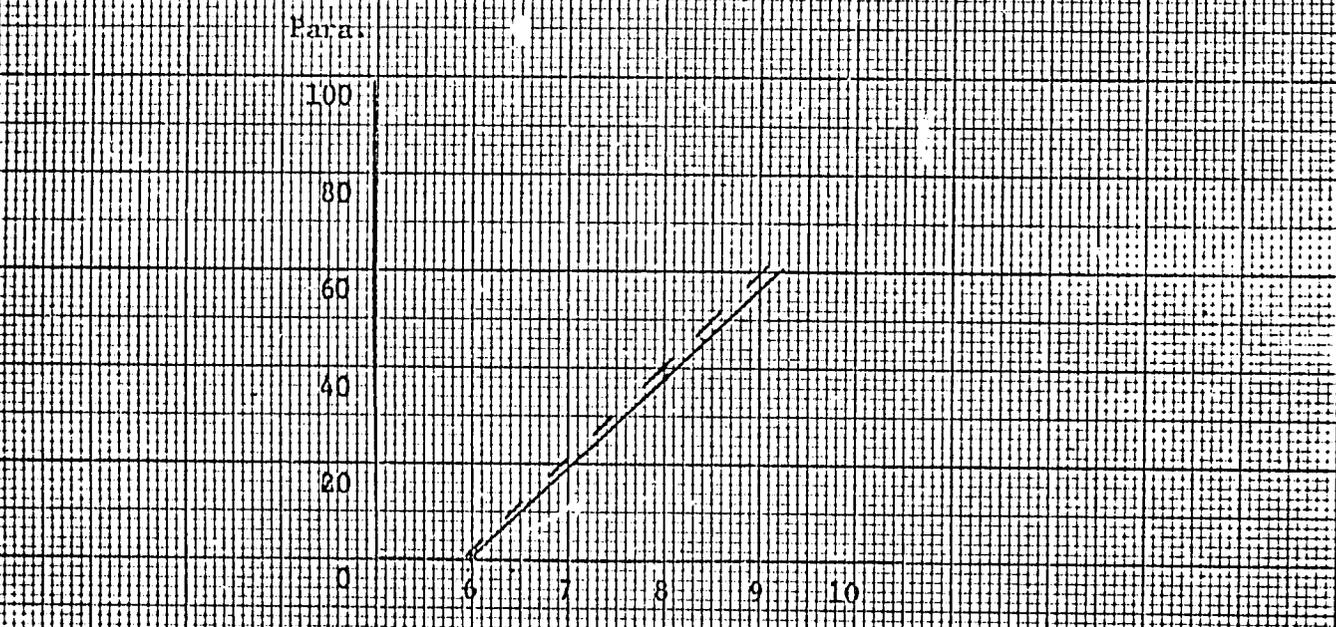
EUGENE DIETZGEN CO.  
MADE IN U. S. A.

140-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH



(4) 40 mg/kg given on day 0

(5) 40 mg/kg given on day 4



(6) 60 mg/kg given on day -4

Control (Not Treated)

Treated

**Table II**  
**Drug Screening Trials Against Babesia rodhaini**

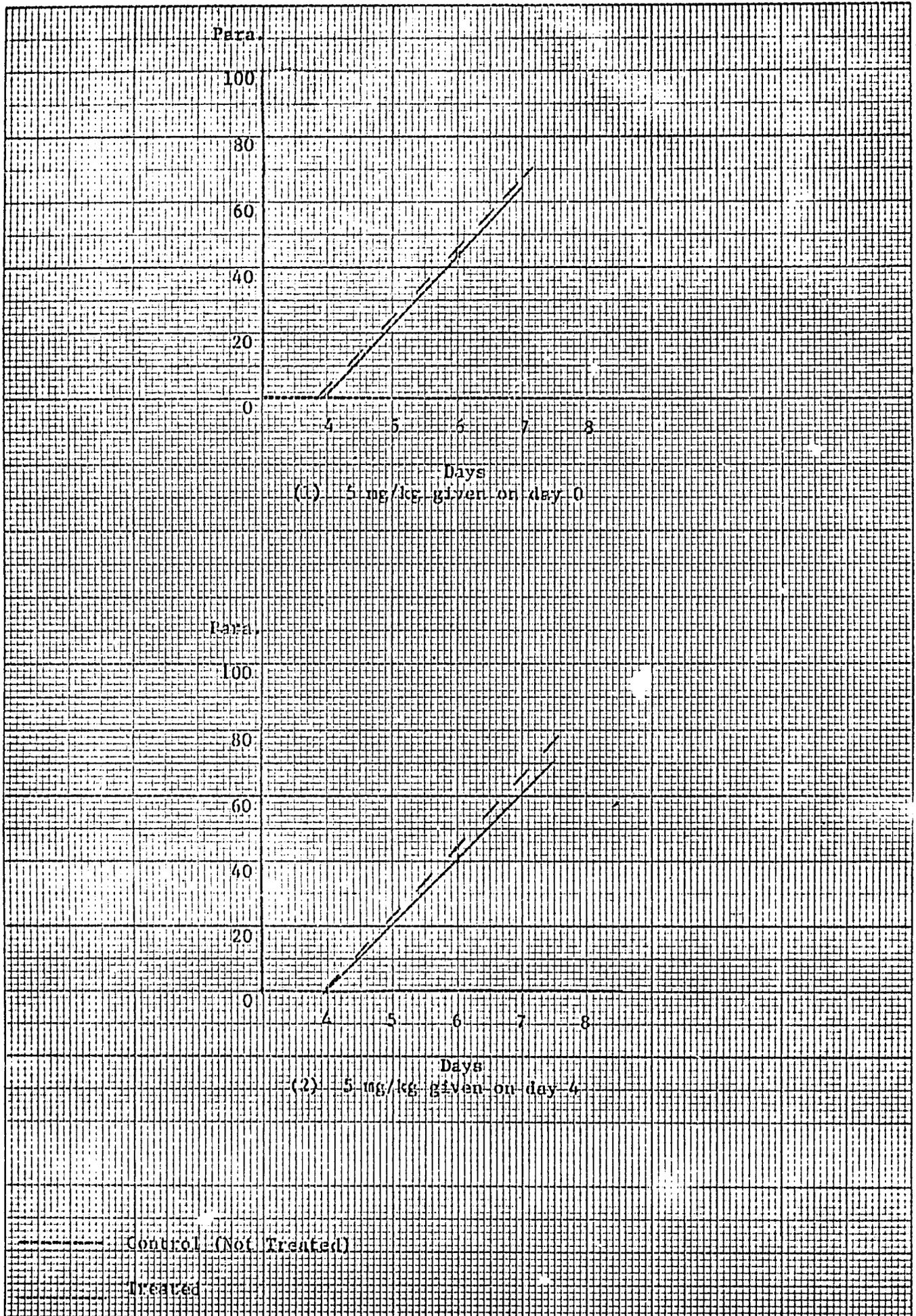
Experimental Drug	i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
NeoA (1) (s/c) 1	8.4 x 10 <sup>5</sup>	0	5 mg/kg	5/5	21.9 ± 4.6	0.86	NS
" " " 2	8.4 x 10 <sup>5</sup>	4	5 mg/kg	4/5	19.8 ± 5.8	1.03	NS
4A65 (HCL) (s/c) 3*	8.4 x 10 <sup>5</sup>	0	2 mg/kg	3/5	11.1 ± *3.0	1.49	P<0.01
" " " 4	8.4 x 10 <sup>5</sup>	4	2 mg/kg	0/5	-0.4 ± 0.2	>61.46	P<0.01
4A65 (dipropionate) 5*	8.4 x 10 <sup>5</sup>	0	2 mg/kg	1/5	7.5 ± *2.5	3.50	P<0.01
" " " 6	8.4 x 10 <sup>5</sup>	4	2 mg/kg	0/5	-0.3 ± 0.6	>61.46	P<0.01
DMSO (s/c) 7	8.4 x 10 <sup>5</sup>	0	0.1 ml	5/5	26.2 ± 3.2	0.81	NS
" " " 8	8.4 x 10 <sup>5</sup>	4	0.1 ml	5/5	24.6 ± 1.5	0.82	NS
Untreated Control 9	8.4 x 10 <sup>5</sup>	-	-----	4/5	21.5 ± 4.3	-----	-----

(1) Neoarsephenamine.

\* Difference does not reach significance.

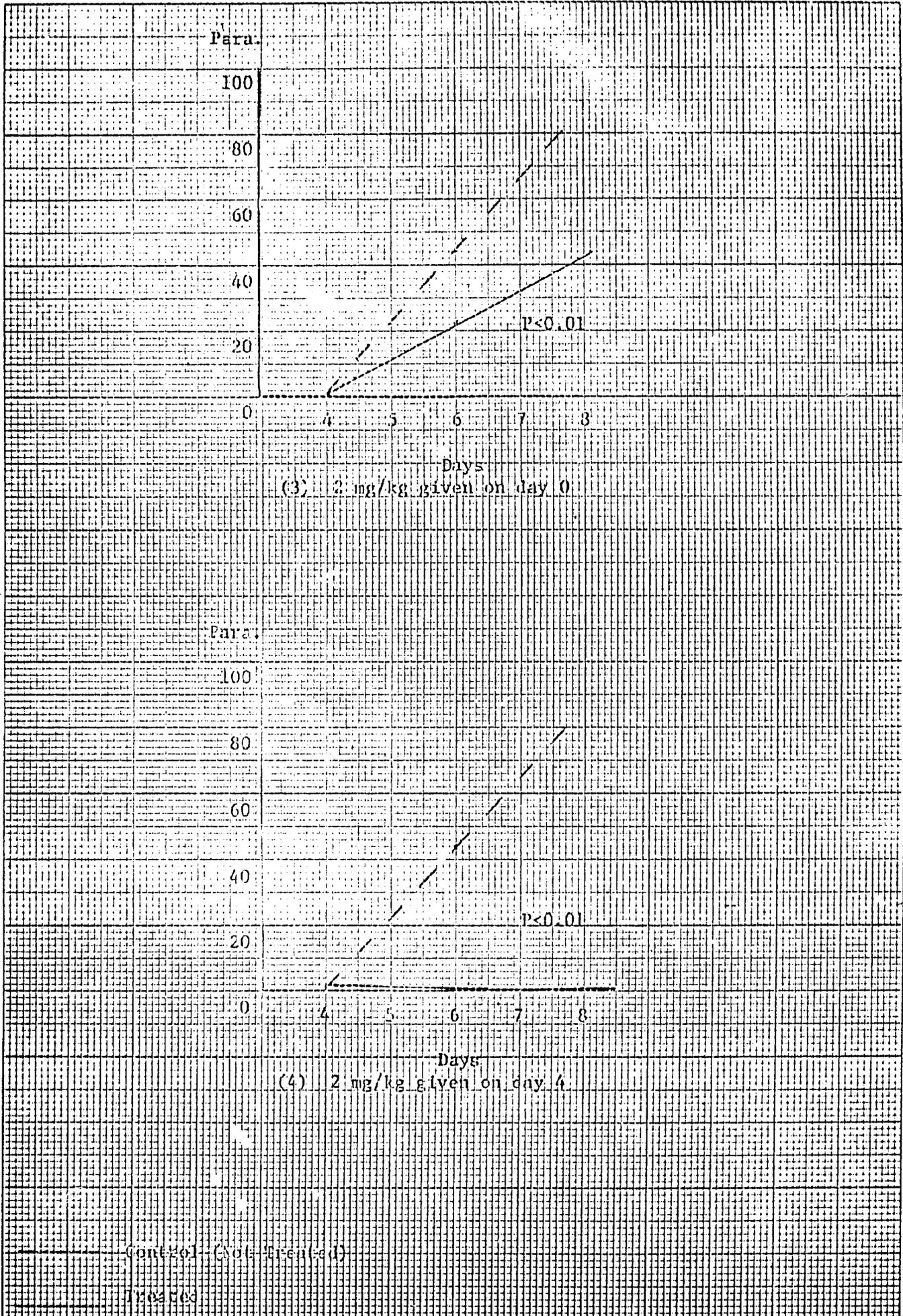
EUGENE DIETZGEN CO.  
MADE IN U. S. A.

NO. 340-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH



EUGENE DIETZGEN CO.  
MADE IN U. S. A.

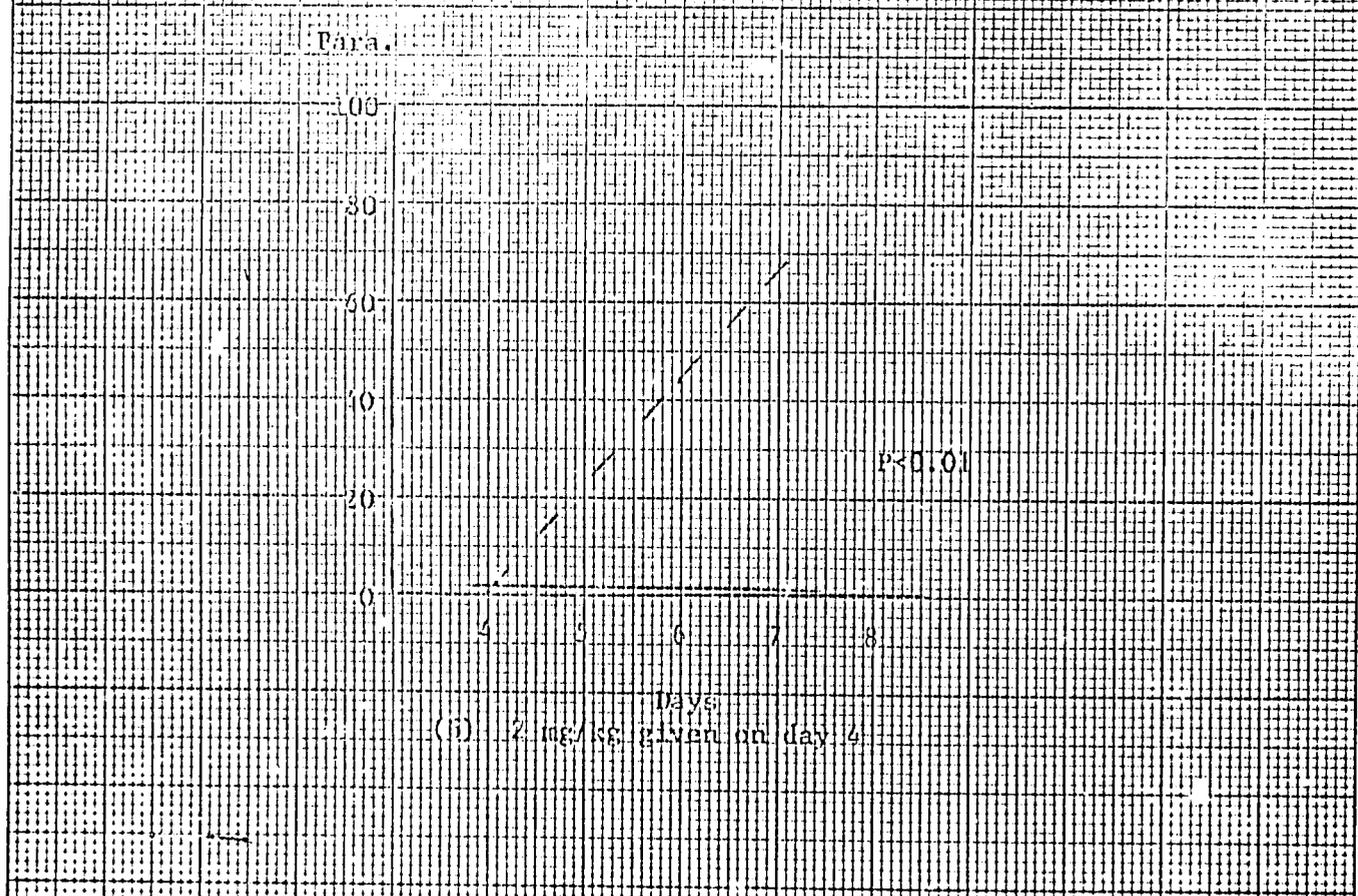
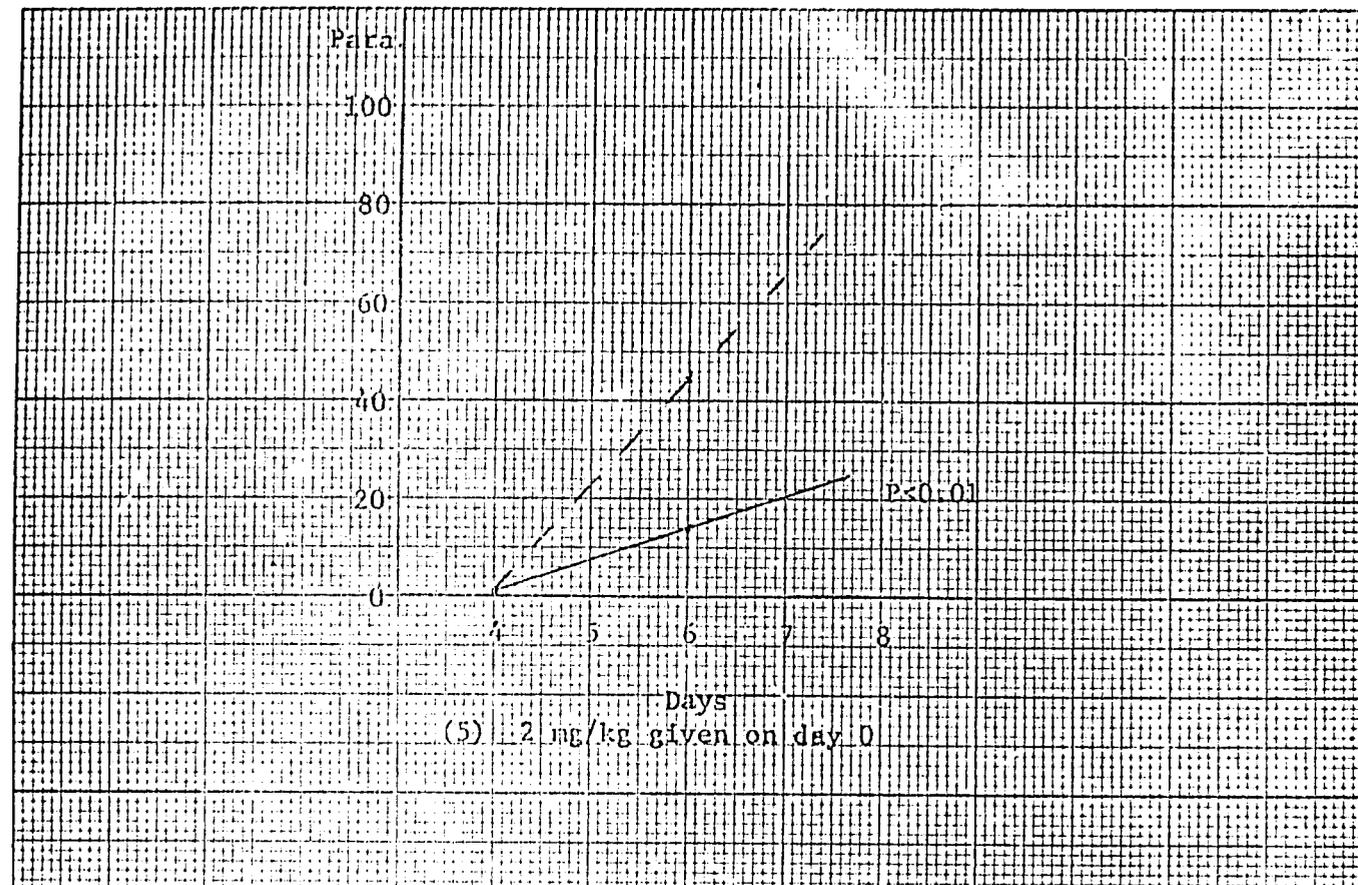
NO. 340-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH



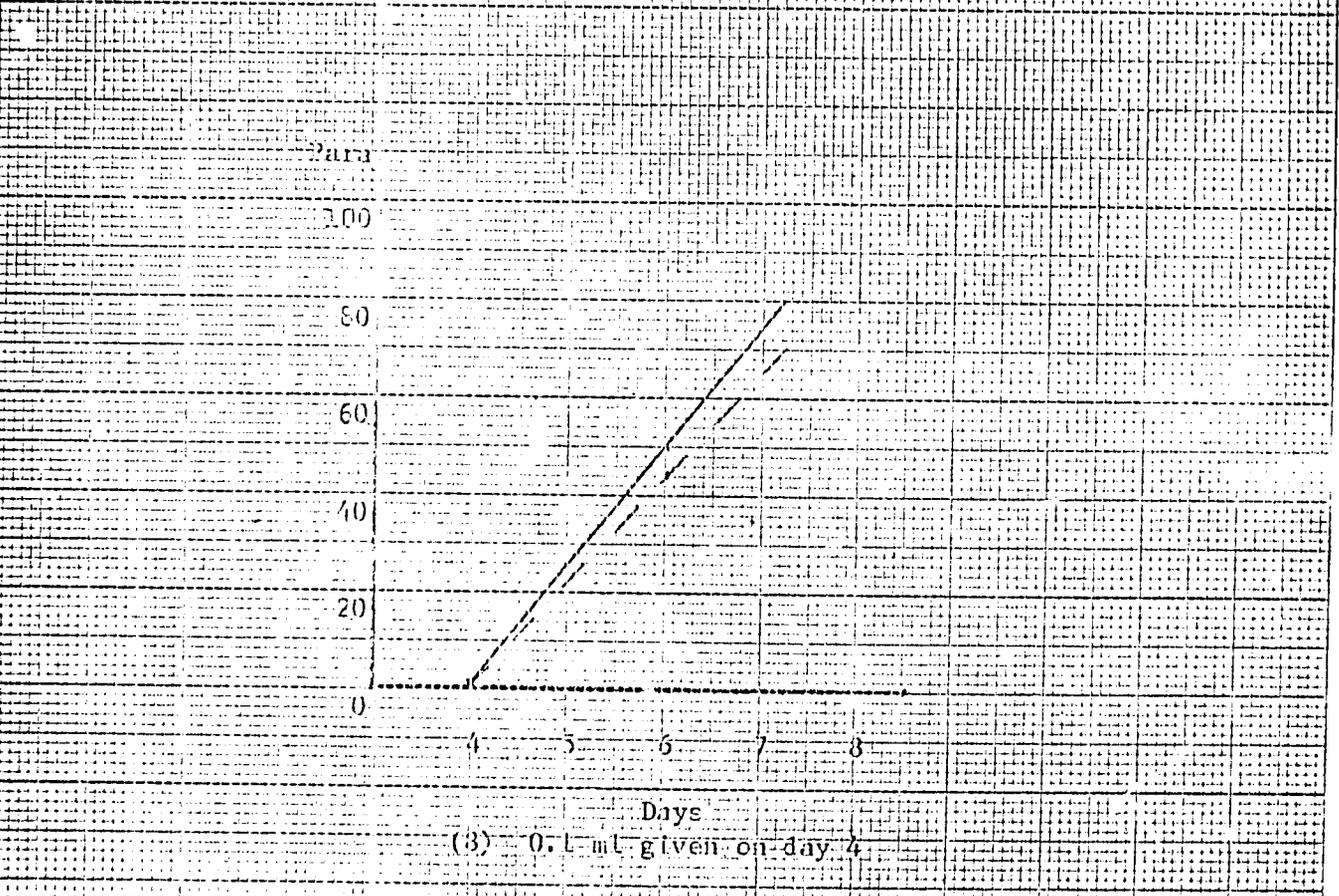
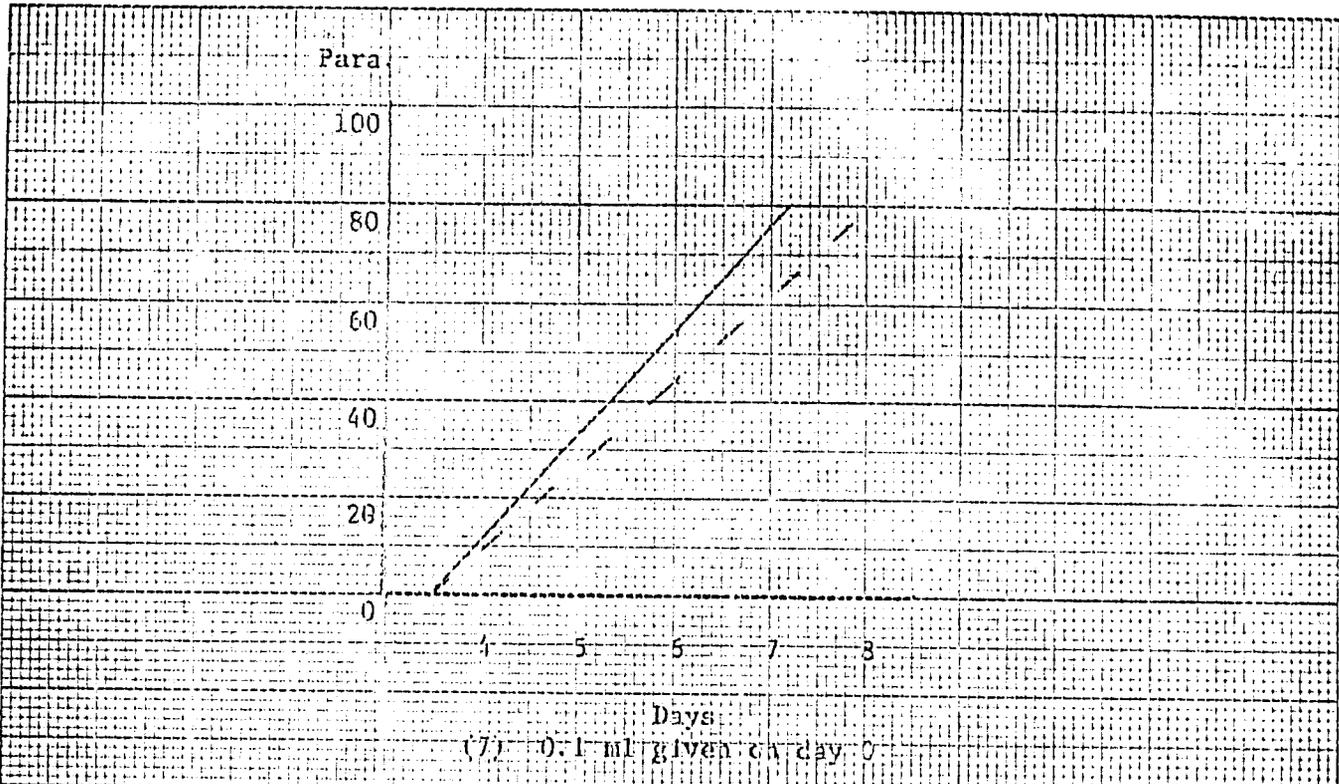
Control (Not Treated)  
Treated

EUBENE DIETZGEN CO.  
MADE IN U. S. A.

340-20 DIETZGEN GRAPH PAPER  
20 X 20 PER INCH



Control (Not Treated)  
Treated



----- Control (Not Treated)

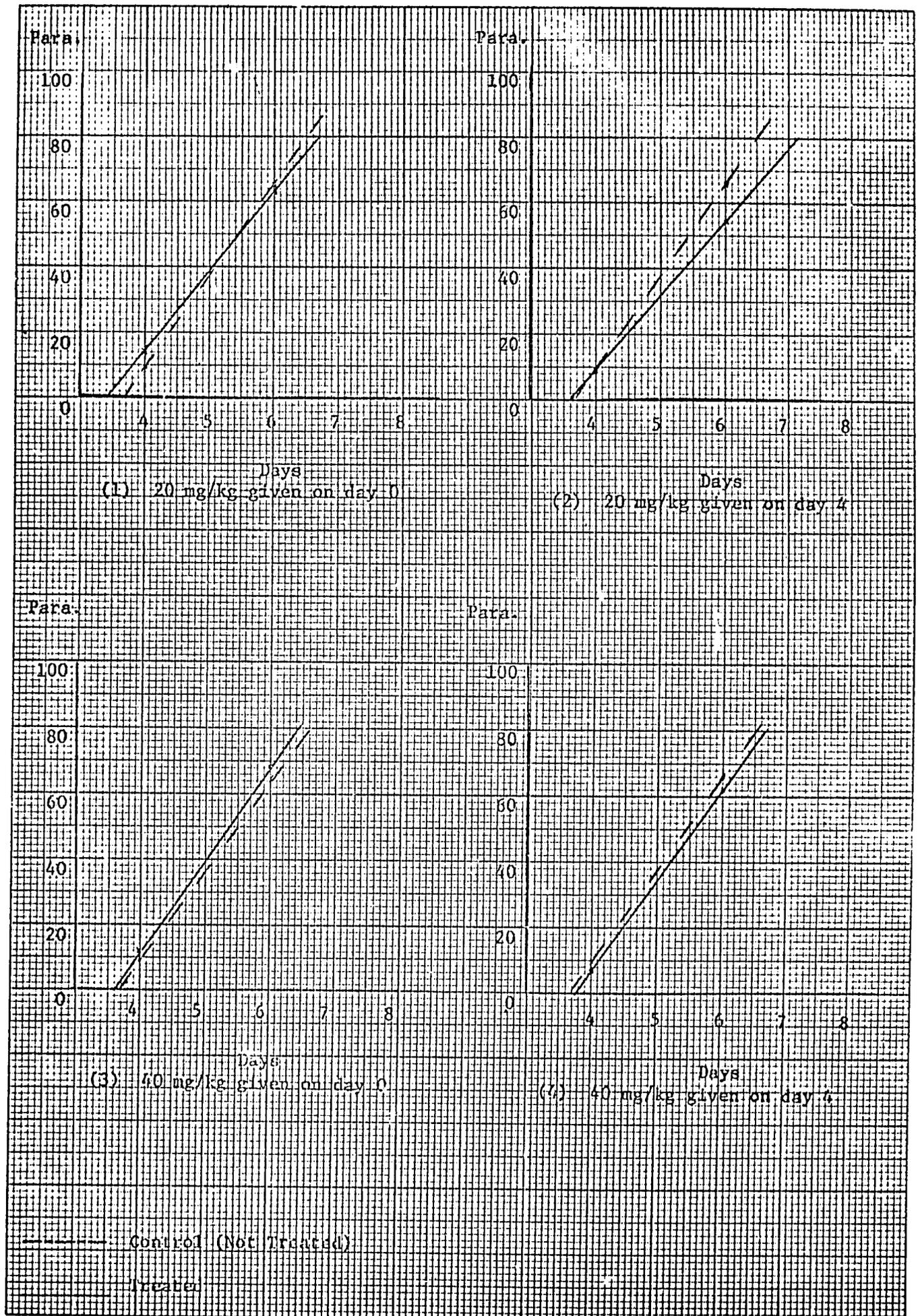
----- Treated

Table 1J

Drug Screening Trials Against Babesia rodhaini

Experimental Drug			i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
7930	(s/c)	1	$5.6 \times 10^5$	0	20 mg/kg	5/5	$24.2 \pm 7.3$	1.04	NS
"	"	2	$5.6 \times 10^5$	4	20 mg/kg	5/5	$22.8 \pm 3.5$	1.06	NS
"	"	3	$5.6 \times 10^5$	0	40 mg/kg	5/5	$28.6 \pm 8.5$	0.98	NS
"	"	4	$5.6 \times 10^5$	4	40 mg/kg	5/5	$27.4 \pm 4.2$	1.00	NS
Oxytet. (1)	(s/c)	5	$5.6 \times 10^5$	0	40 mg/kg	5/5	$23.5 \pm 6.2$	1.05	NS
"	"	6	$5.6 \times 10^5$	4	40 mg/kg	5/5	$21.0 \pm 4.0$	1.09	NS
Untreated Control		7	$5.6 \times 10^5$	-	-----	5/5	$27.4 \pm 5.8$	-----	--

(1) Oxytetracycline.



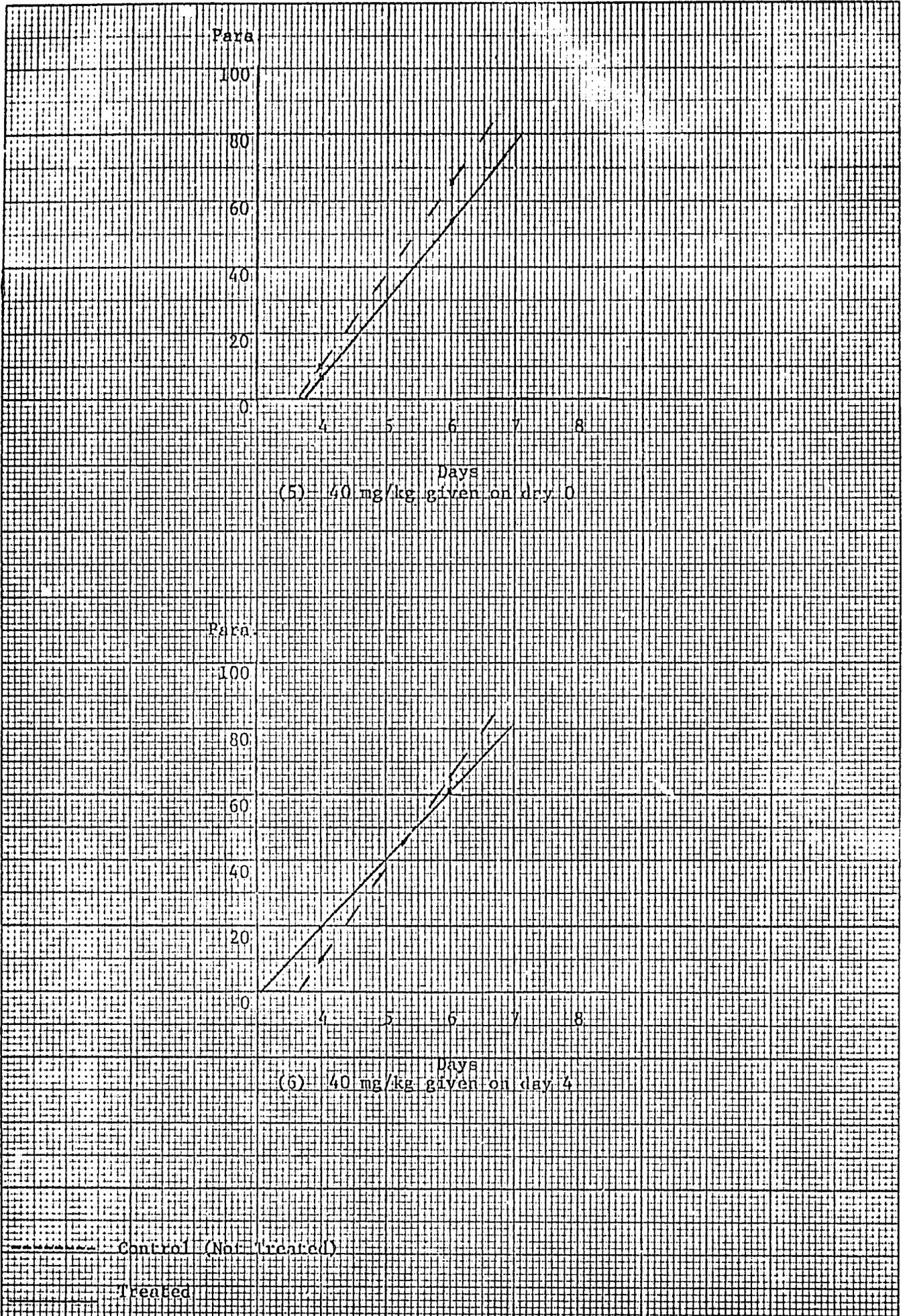


Table 1K

Drug Screening Trials Against Babesia rodhaini

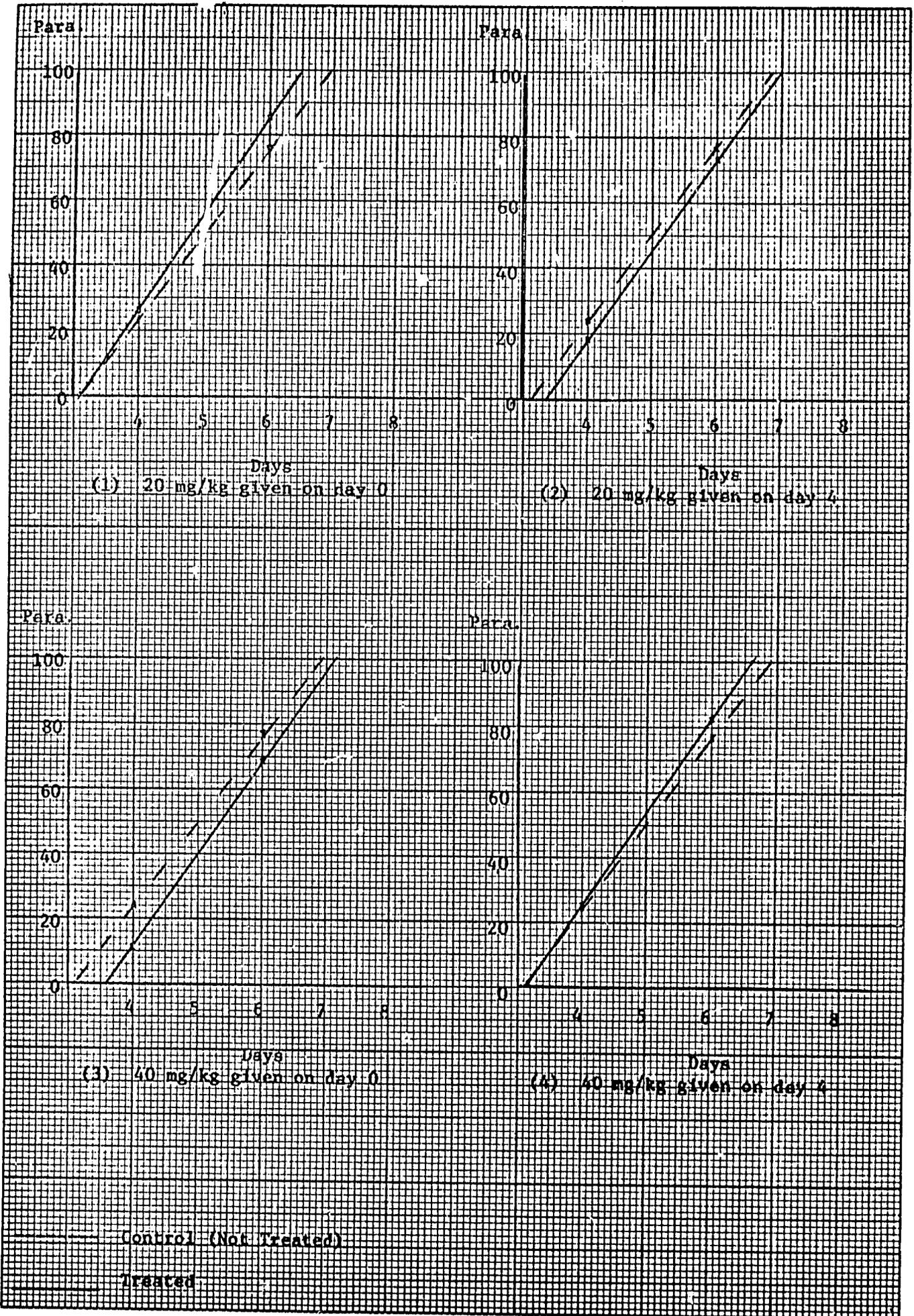
Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
ZE 46212	(s/c) 1	$6.6 \times 10^5$	0	20 mg/kg	5/5	$29.5 \pm 7.2$	0.96	NS
" "	" 2	$6.6 \times 10^5$	4	20 mg/kg	5/5	$27.3 \pm 8.3$	0.99	NS
" "	" 3	$6.6 \times 10^5$	0	40 mg/kg	5/5	$28.3 \pm 13.7$	0.98	NS
" "	" 4	$6.6 \times 10^5$	4	40 mg/kg	4/5	$28.5 \pm 12.5$	0.98	NS
4A65 & DMSO*	(s/c) 5	$6.6 \times 10^5$	0	1 mg/kg*	0/5	0	>88.40	P<0.01
" "	" 6	$6.6 \times 10^5$	4	1 mg/kg*	0/5	$-8.7 \pm 7.2$	>88.40	P<0.01
4A65 & PBS**	(s/c) 7	$6.6 \times 10^5$	0	1 mg/kg**	0/5	0	>88.40	P<0.01
" "	" 8	$6.6 \times 10^5$	4	1 mg/kg**	0/5	$-6.5 \pm 2.8$	>88.40	P<0.01
Untreated Control	9	$6.6 \times 10^5$	-	-----	5/5	$26.7 \pm 10.0$	-----	-----
" "	10	$6.6 \times 10^5$	-	-----	5/5	$38.4 \pm 2.4$	-----	-----

\*4A65 & DMSO - 4A65 was administered in a solution with 50% DMSO.

\*\*4A65 & PBS - 4A65 was administered in a solution with PBS.

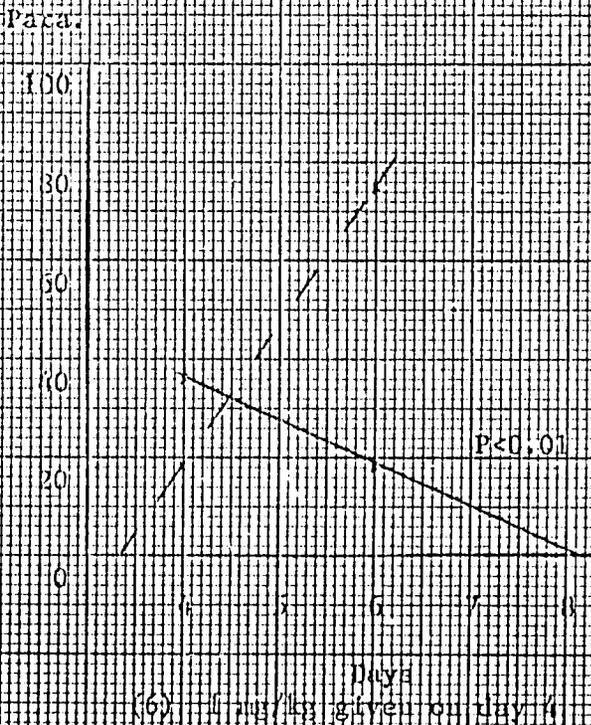
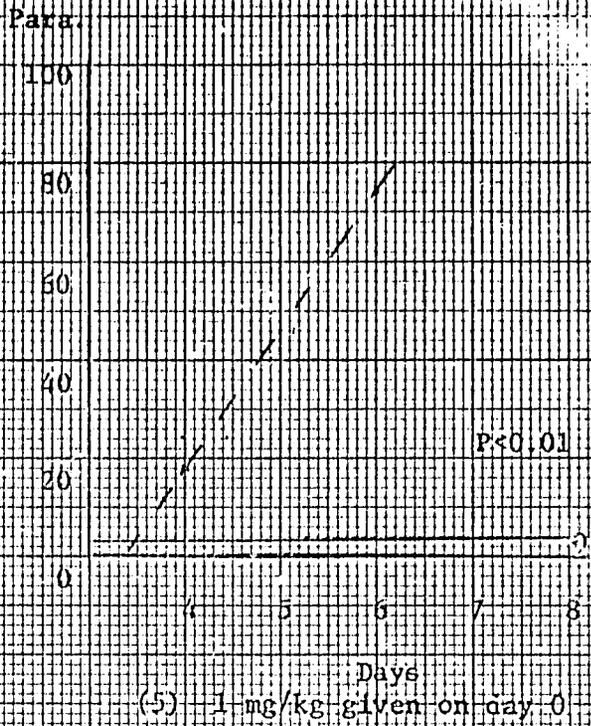
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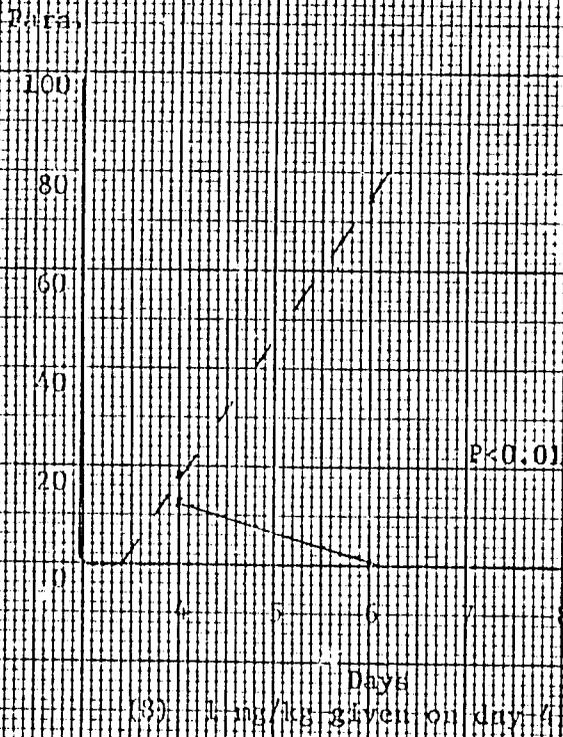
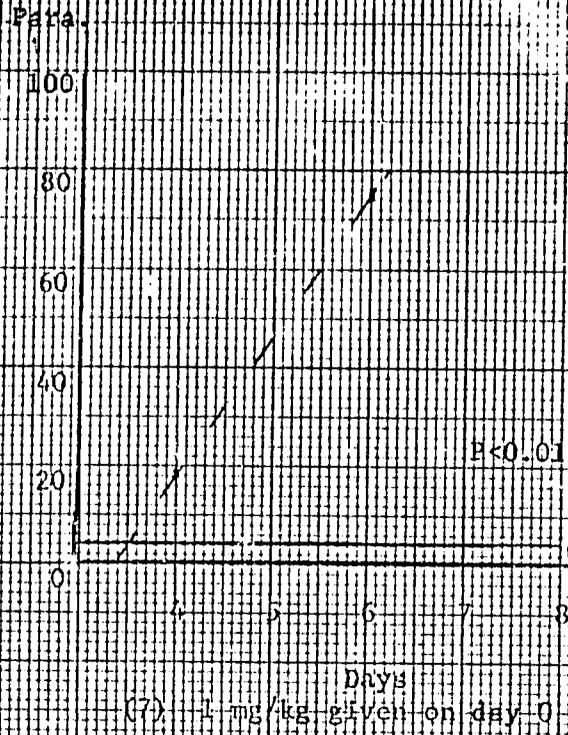


Control (Not Treated)

Treated

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Control (Not Treated)

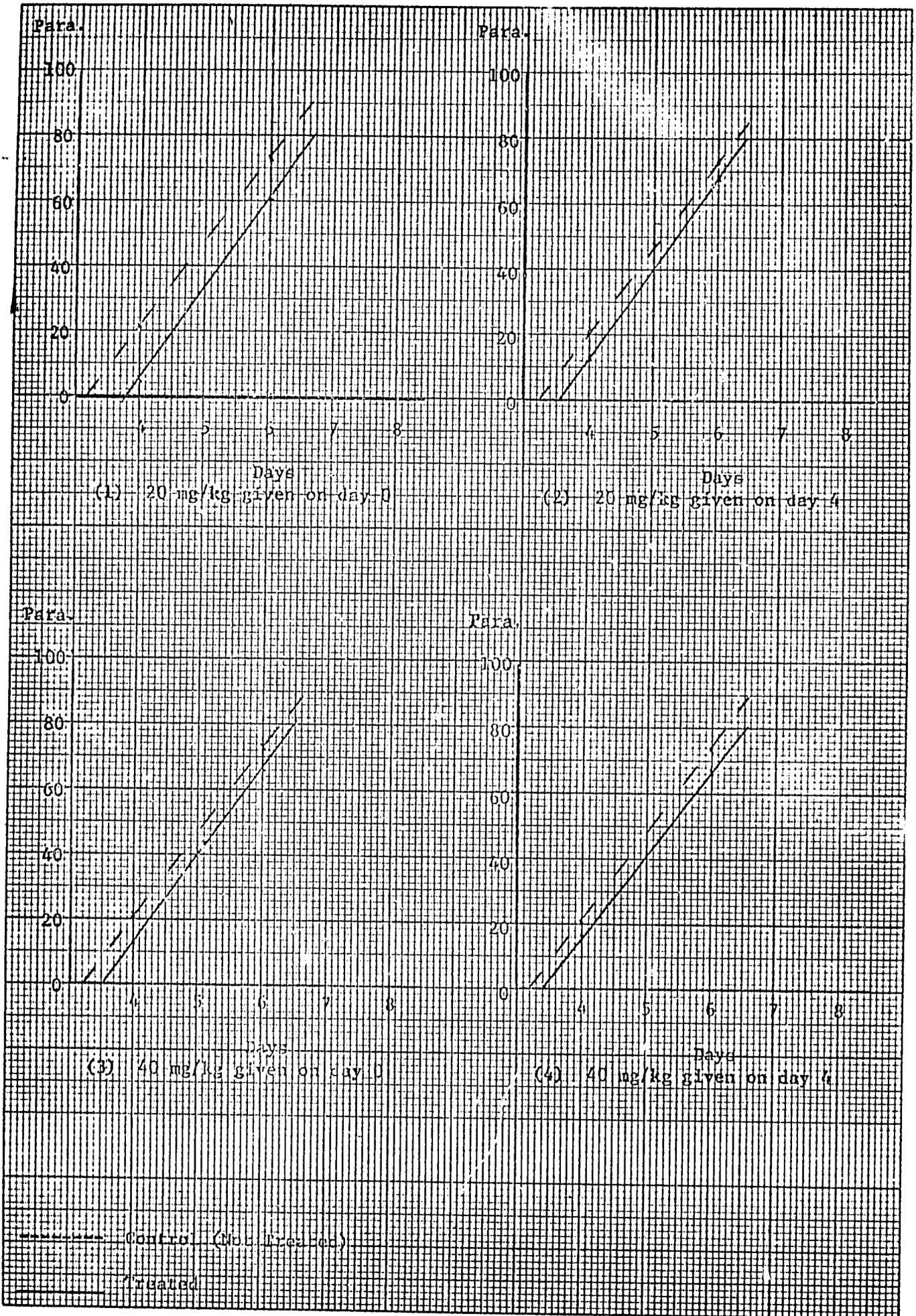
Treated

Table 1L  
Drug Screening Trials Against Babesia rodhaini

Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
AX 26820	(s/c)	1	0	20 mg/kg	4/5	27.8 ± 5.0	1.12	NS
" "	"	2	4	20 mg/kg	5/5	27.9 ± 2.4	0.98	NS
" "	"	3	0	40 mg/kg	4/5	26.3 ± 6.4	1.15	NS
" "	"	4	4	40 mg/kg	4/5	25.6 ± 4.1	1.16	NS
AY 62009	(s/c)	5	0	20 mg/kg	5/5	27.2 ± 3.7	0.98	NS
" "	"	6	4	20 mg/kg	4/5	26.9 ± 3.0	1.14	NS
" "	"	7	0	40 mg/kg	5/5	27.5 ± 1.1	0.98	NS
" "	"	8	4	40 mg/kg	4/5	24.2 ± 2.7	1.18	NS
Untreated Control		9	-	-----	4/4	26.0 ± 5.0	-----	--

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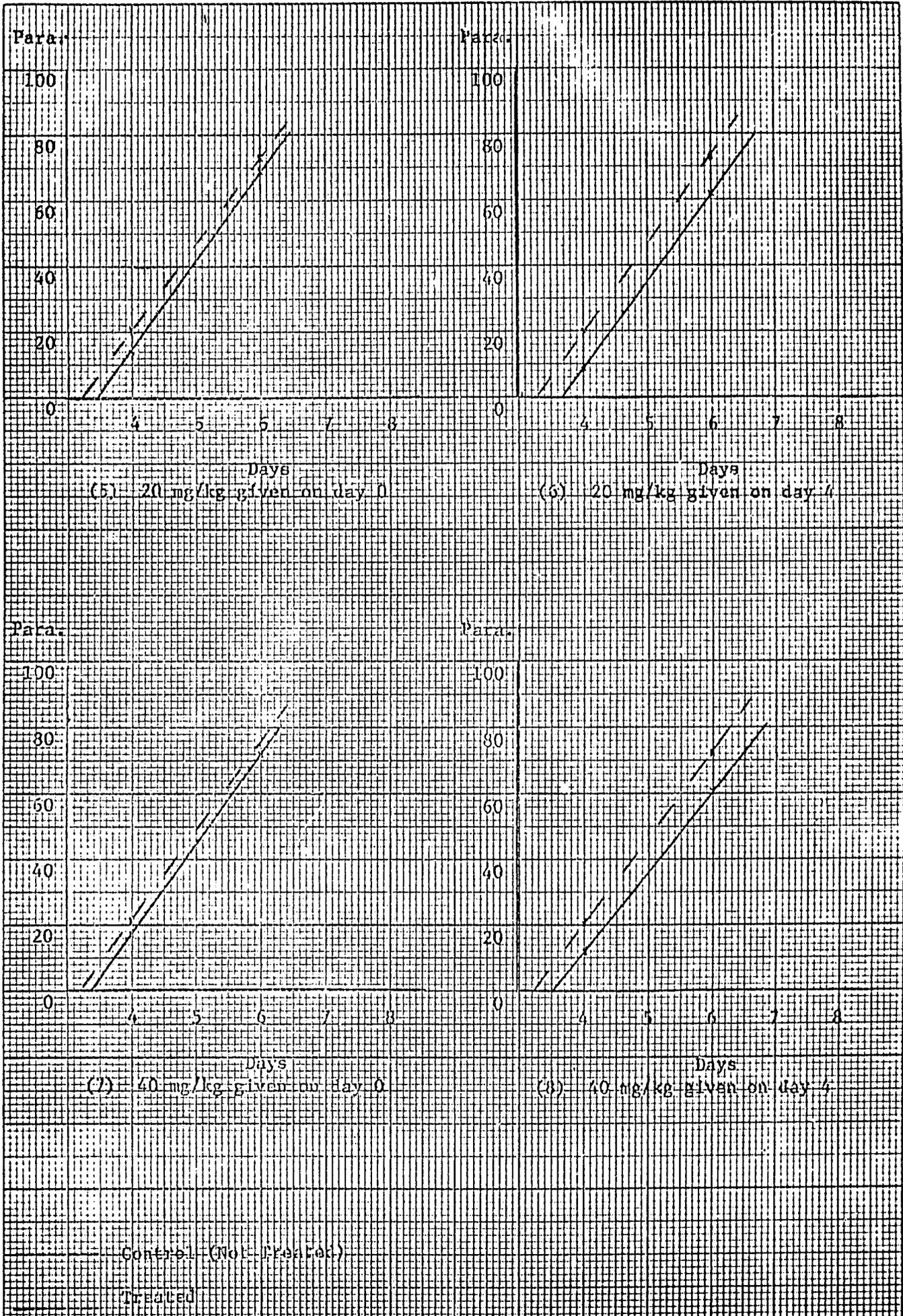
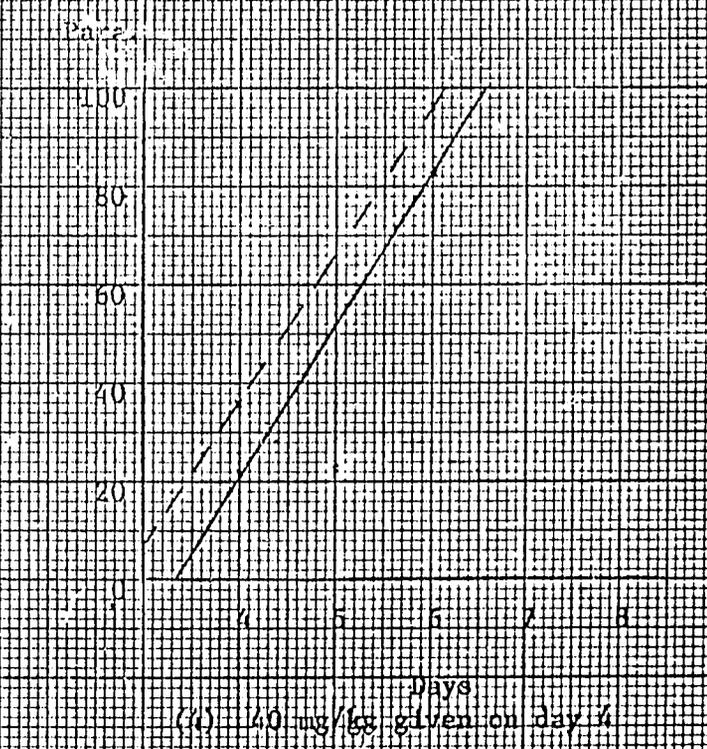
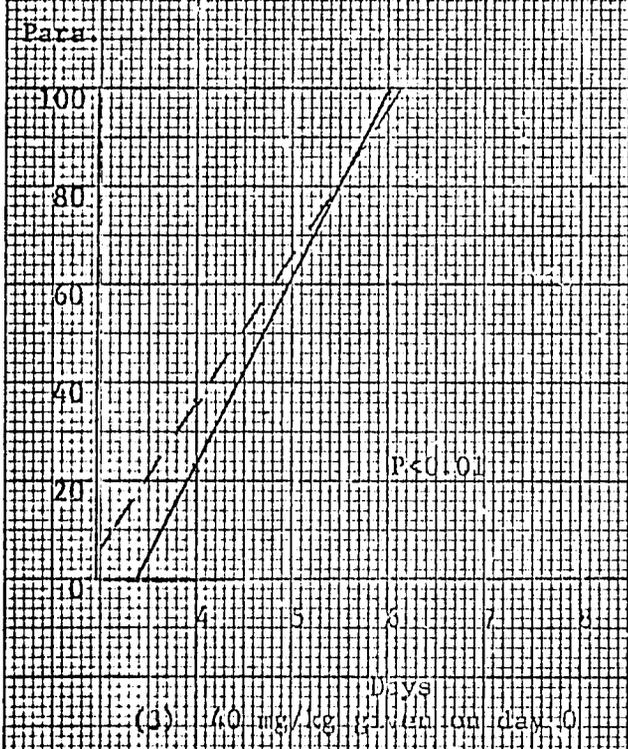
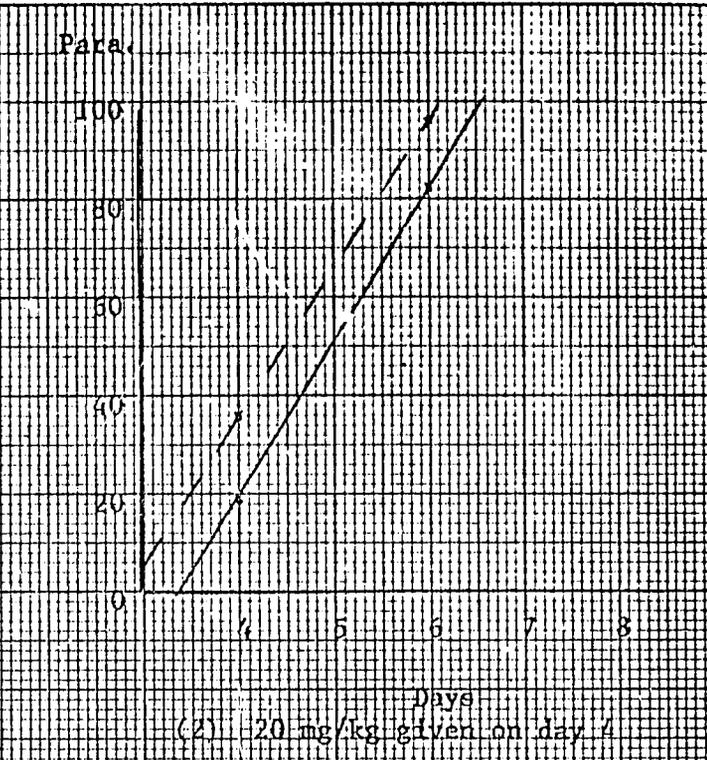
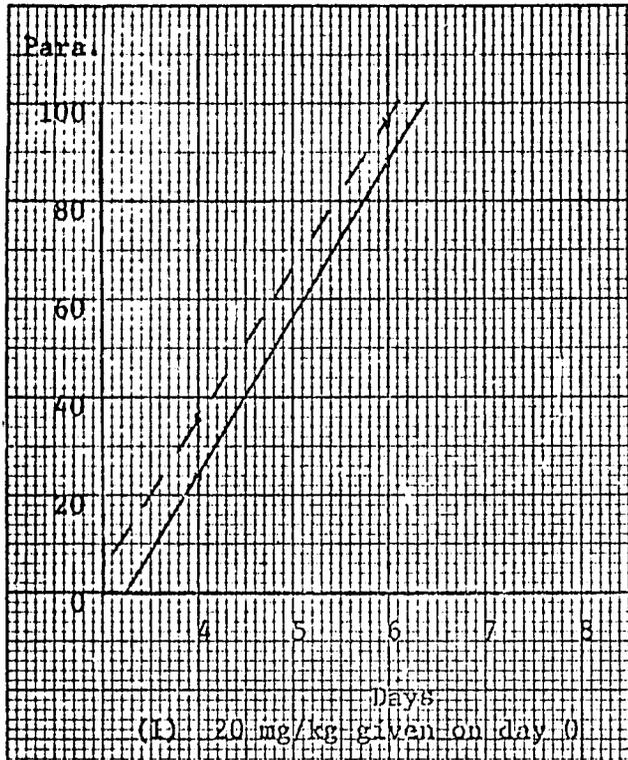


Table 1M  
Drug Screening Trials Against Babesia rodhaini

Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
AG 40405	(s/c)	1	0	20 mg/kg	5/5	32.0 ± 8.0	0.98	NS NS P<0.01 NS
"	"	2	4	20 mg/kg	5/5	31.5 ± 11.1	0.98	
"	"	3	0	40 mg/kg	5/5	37.8 ± 3.5	0.91	
"	"	4	4	40 mg/kg	5/5	29.9 ± 5.1	1.00	
AG 74492	(s/c)	5	0	20 mg/kg	0/5	0	>80.00	P<0.01 P<0.01 P<0.01 P<0.01
"	"	6	4	20 mg/kg	0/5	-9.6 ± 2.2	>80.00	
"	"	7	0	40 mg/kg	0/5	0	>80.00	
"	"	8	4	40 mg/kg	0/5	-7.3 ± 4.5	>80.00	
Untreated Control		9	-	-----	5/5	30.0 ± 2.3	-----	-----

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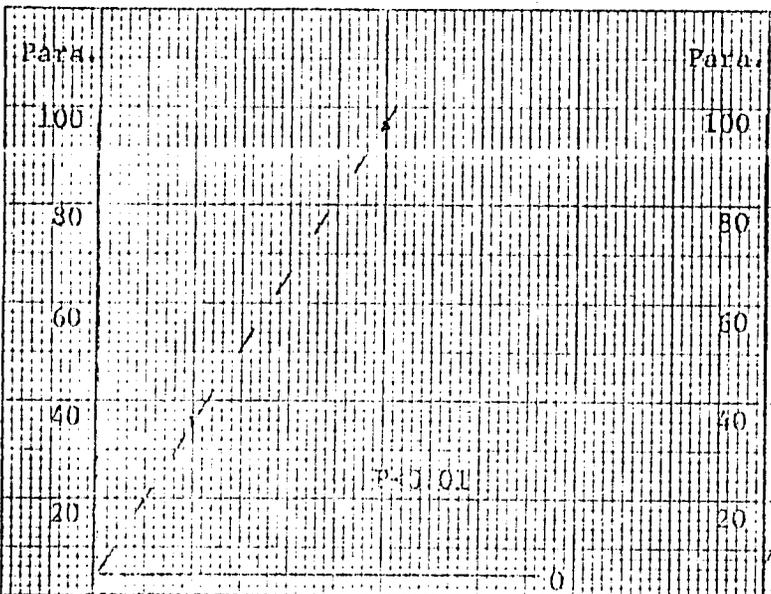


Control (Not Treated)

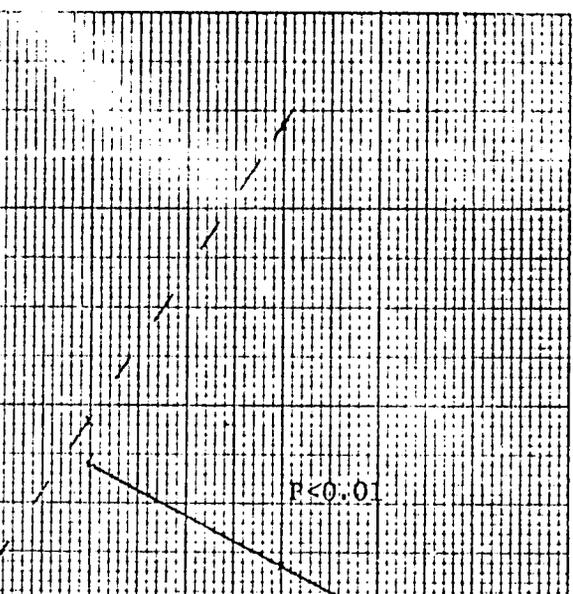
Treated

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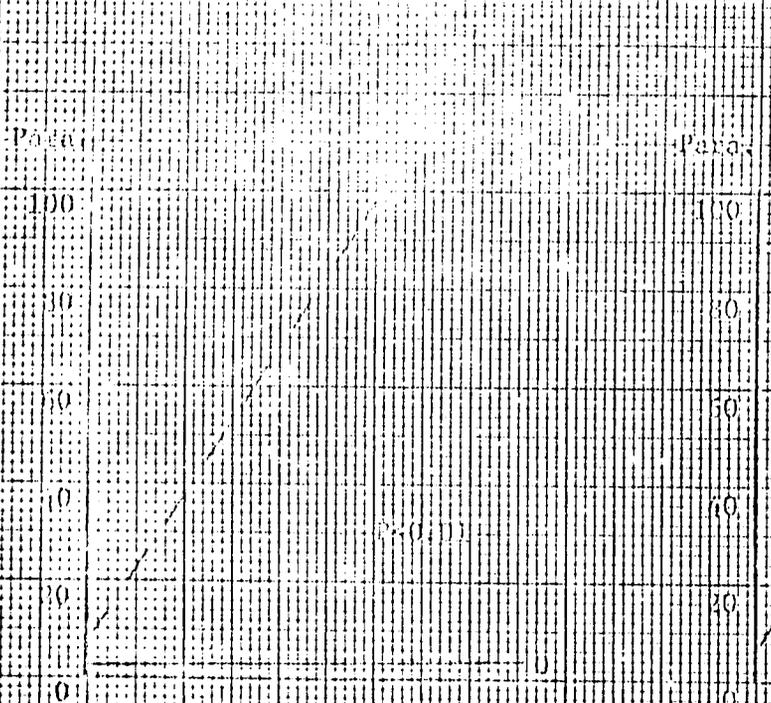
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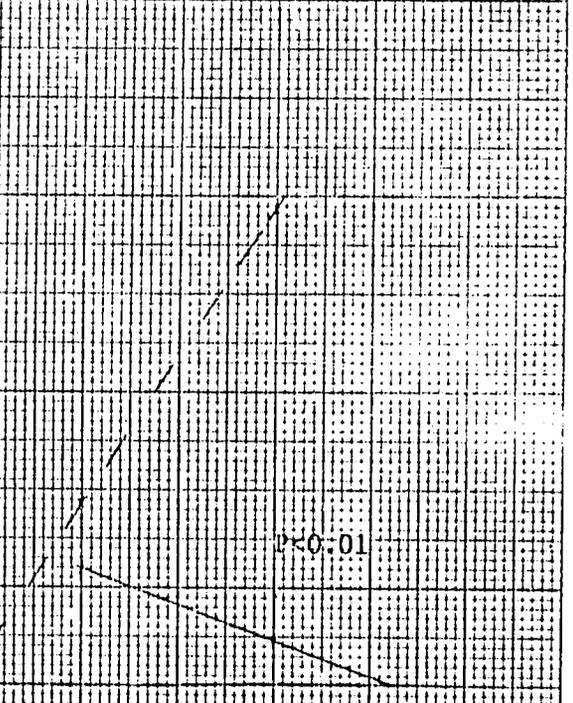
Days  
(5) 20 mg/kg glycyrrhizin on day 0



Days  
(6) 20 mg/kg glycyrrhizin on day 4



Days  
(7) 40 mg/kg glycyrrhizin on day 0



Days  
(8) 40 mg/kg glycyrrhizin on day 4

Control (No Glycyrrhizin)

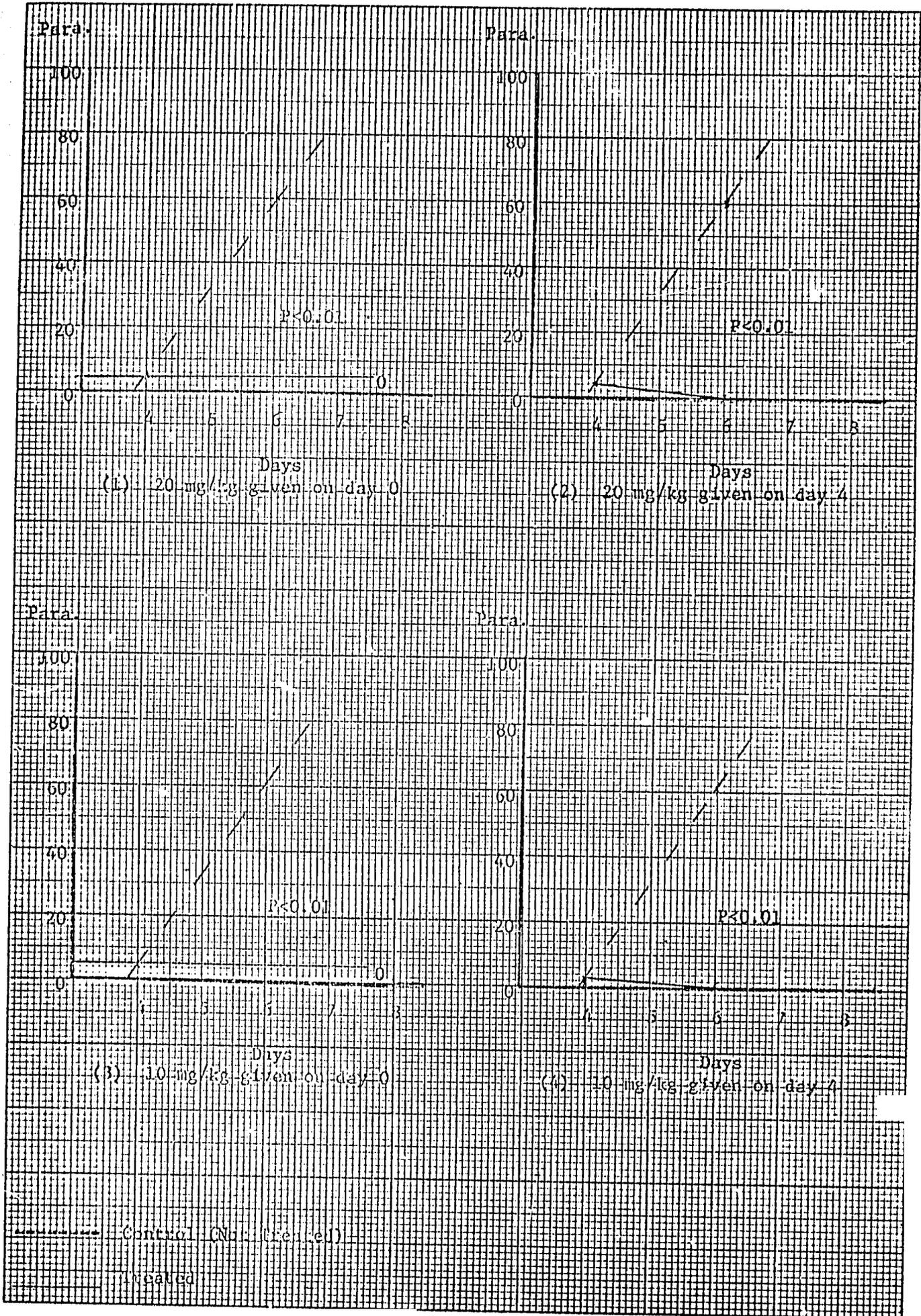
Control (No Glycyrrhizin)

Table IN  
Drug Screening Trials Against Babesia rodhaini

Experimental Drug	i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
AG 74492 (s/c) 1	$5.9 \times 10^5$	0	20 mg/kg	0/5	0	>77.60	P<0.01
" " " 2	$5.9 \times 10^5$	4	20 mg/kg	0/5	$-3.2 \pm 5.3$	>77.60	P<0.01
" " " 3	$5.9 \times 10^5$	0	10 mg/kg	0/5	0	>77.60	P<0.01
" " " 4	$5.9 \times 10^5$	4	10 mg/kg	0/5	$-2.8 \pm 3.5$	>77.60	P<0.01
" " " 5	$5.9 \times 10^5$	0	5 mg/kg	0/5	0	>77.60	P<0.01
" " " 6	$5.9 \times 10^5$	4	5 mg/kg	1/5	$0.5 \pm 12.1$	7.40	P<0.01
" " " 7	$5.9 \times 10^5$	0	2 mg/kg	3/5	$12.0 \pm 8.1$	1.85	P<0.01
" " " 8	$5.9 \times 10^5$	4	2 mg/kg	3/5	$14.0 \pm 5.5$	1.76	P<0.01
" " " 9	$5.9 \times 10^5$	0	1 mg/kg	4/5	$20.1 \pm 5.9$	1.29	P<0.05
" " " 10	$5.9 \times 10^5$	4	1 mg/kg	5/5	$19.2 \pm 3.3$	1.12	P<0.01
Untreated Control 11	$5.9 \times 10^5$	-	-----	5/5	$27.6 \pm 3.2$	-----	-----

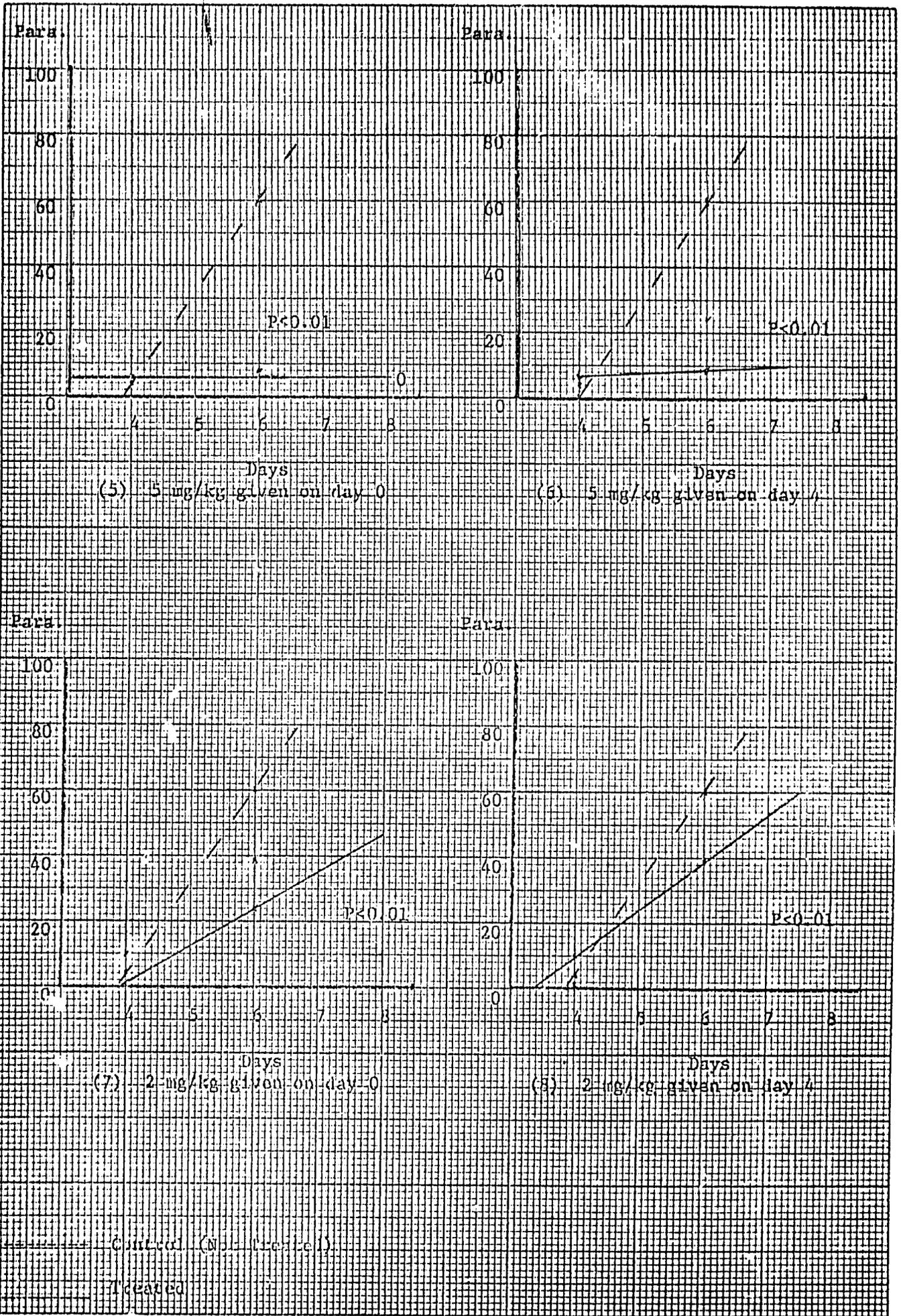
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Para.

100

80

60

40

20

0

4 5 6 7 8

Days

(9) 1 mg/kg given on day 0

$P < 0.05$

Para.

100

80

60

40

20

0

4 5 6 7 8

Days

(10) 1 mg/kg given on day 4

$P < 0.01$

Control (Not Treated)

Treated

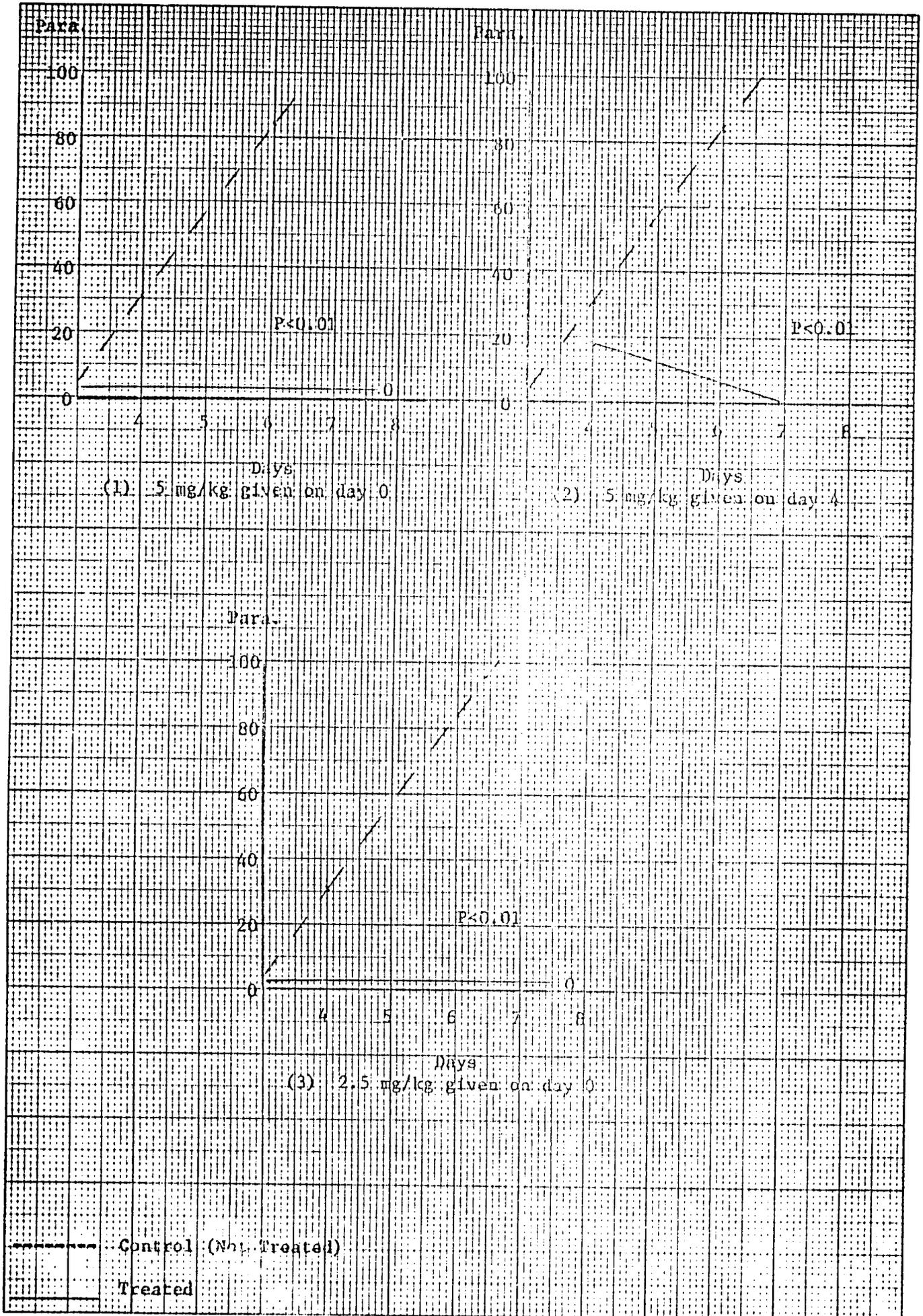
Table 10

Drug Screening Trials Against Babesia rodhaini

Experimental Drug		i/p Chal. On Day 0	Time of Treatment Day	Dose Rate	No. of Deaths	Progressive Parasitemia	Therapeutic Index	Sig.
4A65	(s/c) 1	$5.0 \times 10^5$	0	5.00 mg/kg	0/5	0	>66.70	P<0.01
"	" 2	$5.0 \times 10^5$	4	5.00 mg/kg	0/5	$-5.5 \pm 2.6$	>66.70	P<0.01
"	" 3	$5.0 \times 10^5$	0	2.50 mg/kg	0/5	0	>66.70	P<0.01
"	" 4	$5.0 \times 10^5$	4	2.50 mg/kg	0/5	$-4.1 \pm 1.5$	>66.70	P<0.01
"	" 5	$5.0 \times 10^5$	0	1.00 mg/kg	0/5	0	>66.70	P<0.01
"	" 7	$5.0 \times 10^5$	0	0.50 mg/kg	3/5	$7.1 \pm 4.5$	1.80	P<0.05
"	" 8	$5.0 \times 10^5$	4	0.50 mg/kg	4/5	$21.9 \pm 12.7$	1.08	NS
"	" 9	$5.0 \times 10^5$	0	0.25 mg/kg	4/5	$23.1 \pm 7.3$	1.06	NS
"	" 10	$5.0 \times 10^5$	4	0.25 mg/kg	3/5	$17.7 \pm 13.4$	1.40	NS
Untreated Control	11	$5.0 \times 10^5$	-	-----	4/5	$26.7 \pm 12.0$	-----	-----

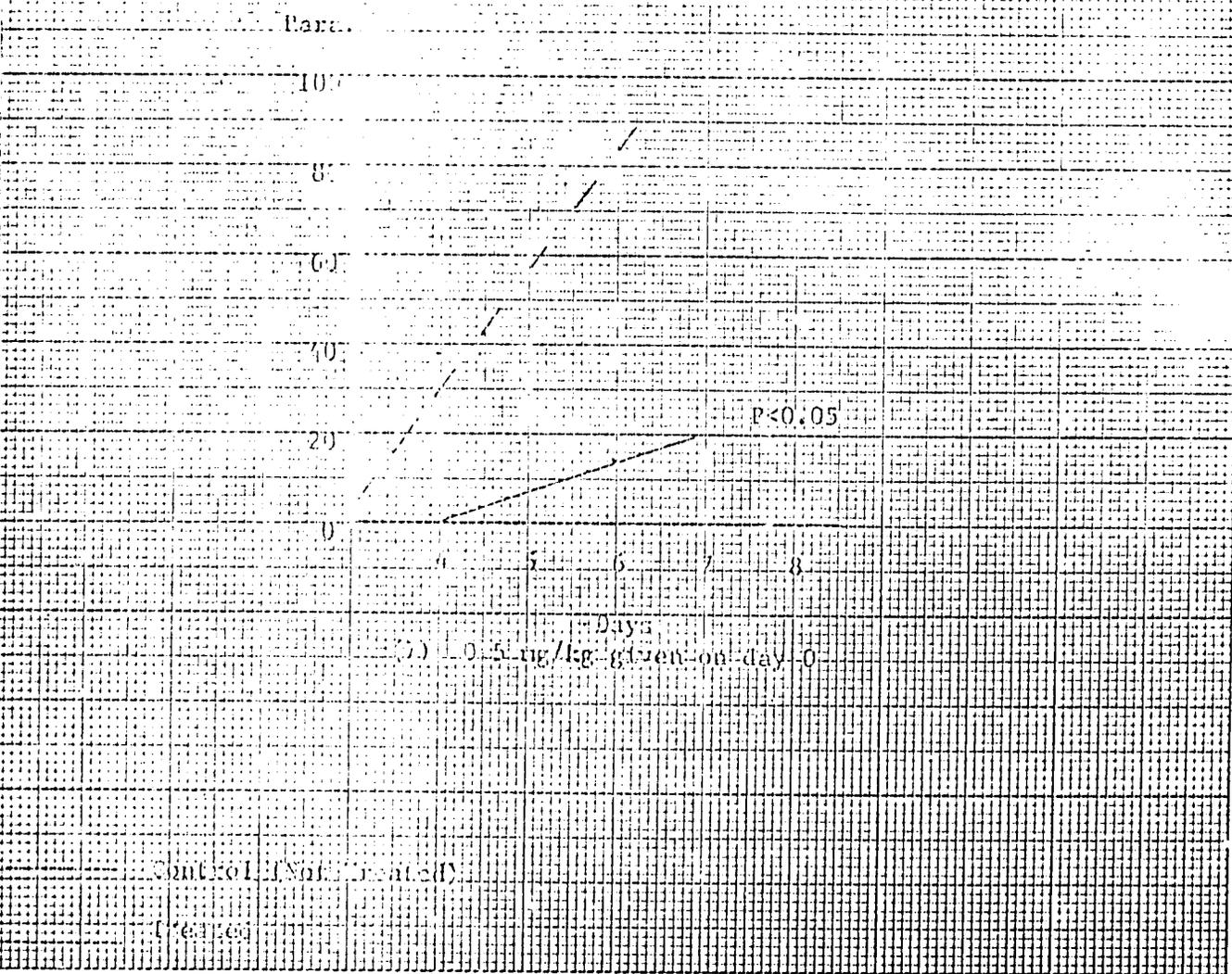
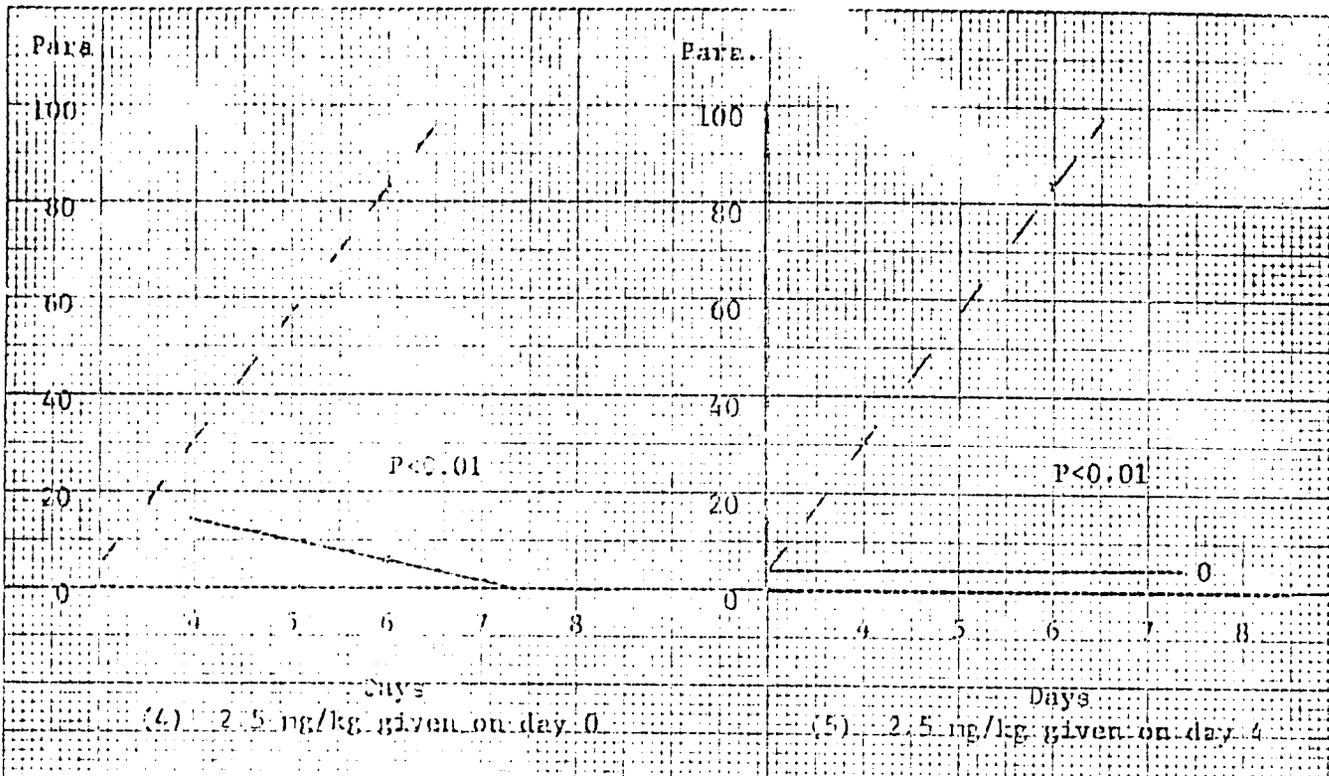
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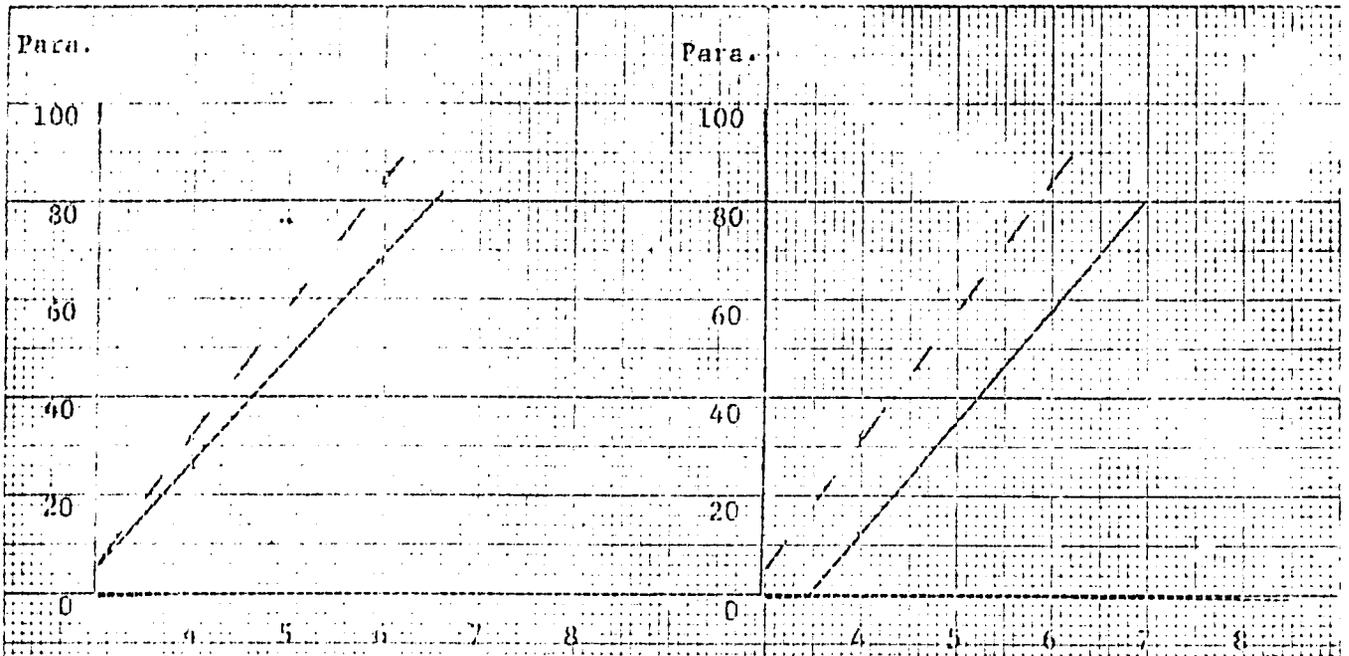
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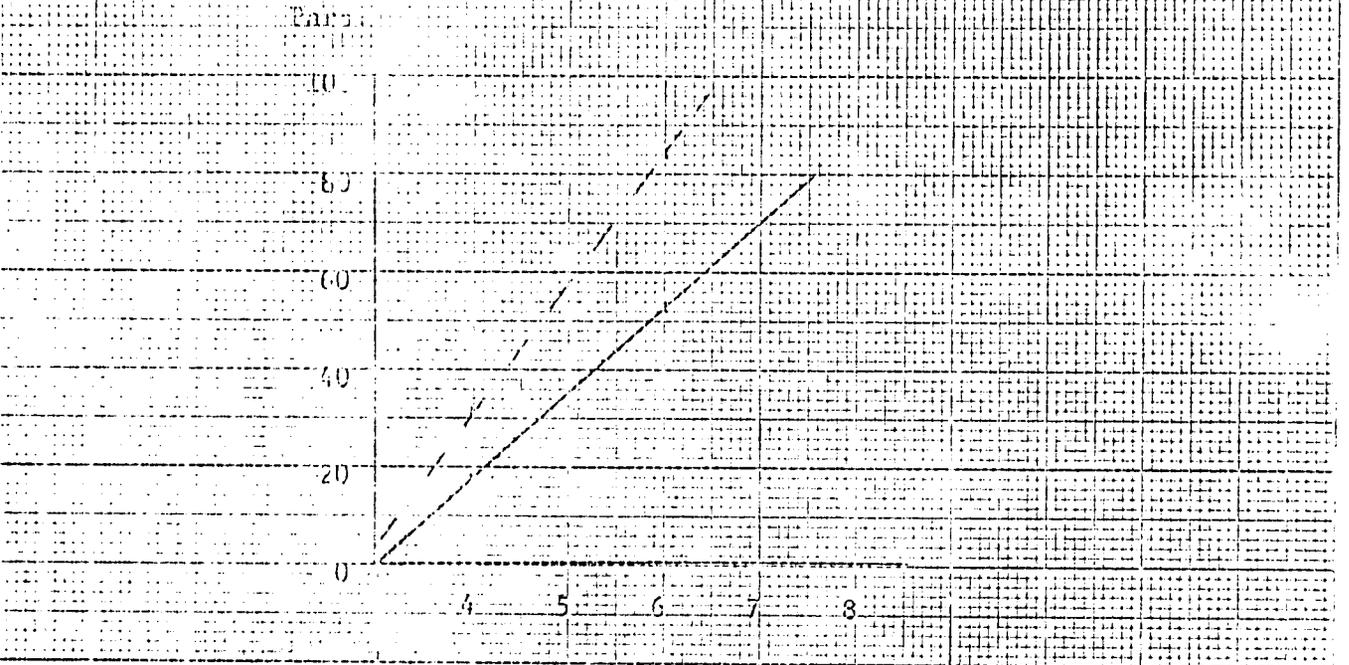
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(8) 5.5 mg/kg given on day 4

(9) 0.25 mg/kg given on day 0



(10) 0.25 mg/kg given on day 4

----- Control (Not Treated)

\_\_\_\_\_ Treated

Table 2

## Description of Compounds Tested

4A65: 3, 3'-Bis-(2-imidazolin-2 yl)-carbanilide dihydrochloride - and dipropionate.

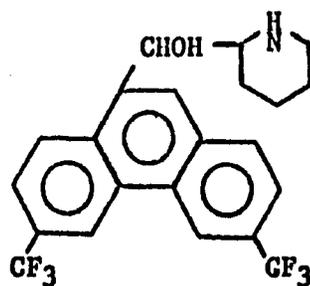
DMSO: Dimethyl sulfoxide.

Poly I/C: Polyriboinosinic Acid and Polyribocytidylic Acid.

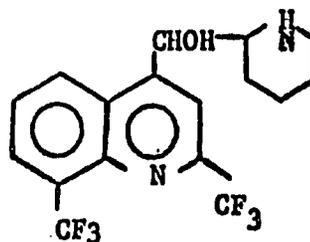
Neoarsephenamine: Sod. 3,3'-diamino-4,4'-dihydroxy arsenobenzene-N-methanol sulfoxylate.

Oxytetracycline: Pfizer's Liquamycin.

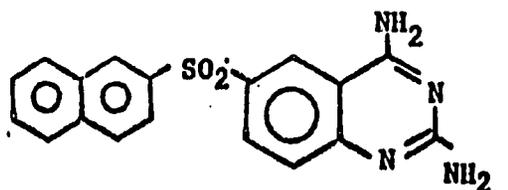
AV-99065  
WR-122455



AX-23187  
WR-142490



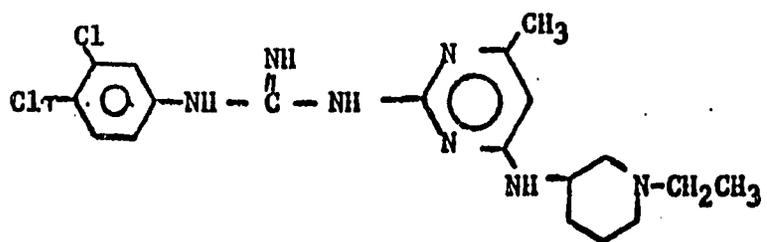
AX-63047  
WR-158122



AB-34313  
WR-87781

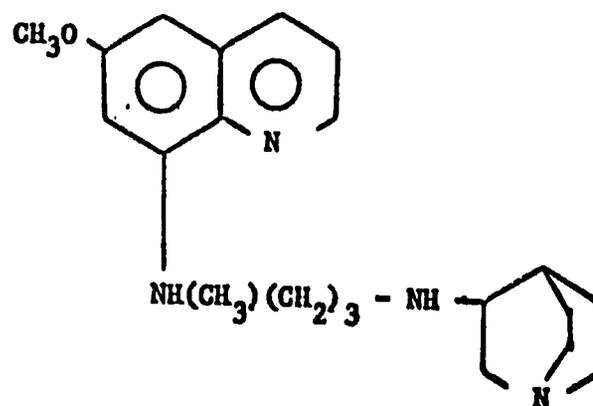
**Minocycline (Lederle)**

AD-44560  
WR-81844

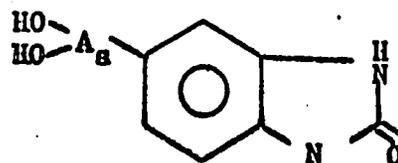




AX-26820  
(WR-161085)



AY-62009  
(WR-152684)



ZE-46212  
(WR-100553)

Doxycycline . HCl

Unsuccessful Attempts to Establish Cattle Babesia  
Infections in White-tailed Deer (Odocoileus virginianus)

Presented at the Annual Wildlife Disease Conference - August 1971

Published in Journal of Wildlife Diseases

K. L. Kuttler, D.V.M.\* O. H. Graham, Ph.D.,\*\* S. R. Johnson, B.S.\* and J. L. Trevino,\*\*

Cattle babesiosis, while no longer occurring in the United States, is a major disease problem in many tropical and sub-tropical areas of the world. This disease is usually associated with one or more ticks of the genus Boophilus. The eradication of these ticks from the U.S. was accompanied by the elimination of bovine babesiosis. Both B. annulatus and bovine Babesia are known to occur in Mexico adjacent to the U.S. border. Because of the proximity of both vector and disease agent to the U.S. intensive surveillance and strict import regulations including the use of adequate acaricides on all animals being moved across the border are mandatory. The regulation of wildlife movement is, however, impractical and at present almost impossible. White-tailed deer are a recognized host for B. annulatus (1). In recent years B. annulatus adults have been recovered from white-tailed deer in Texas as much as 64 kilometers north of the

\* Institute of Tropical Veterinary Medicine, Texas A&M University, College Station, Texas, 77843.

\*\* Entomology Research Division, A.R.S., U.S.D.A., Kerrville, Texas, 78028.

This research was done at a field laboratory operated by the United States Department of Agriculture in Nuevo Laredo, Tamaulipas, Mexico and was made possible by a grant from the U.S. Agency for International Development to the Institute of Tropical Veterinary Medicine, Texas A&M University.

Mexican border,\* emphasizing the possibilities of tick re-introduction by this means.

Spindler, et al. (4) in 1958 reported finding a Babesia like organism in blood films of white-tailed deer which was morphologically similar to B. bigemina. In 1968 Emerson and Wright (3) reported the isolation of Babesia cervi in deer from East Texas. This Babesia was morphologically similar to B. divergens, but could not be propagated in calves, and appeared host specific for deer.

Callow (2) has reported transmission of B. bigemina to sheep and goats by B. microplus and the re-infection of B. microplus with B. bigemina from these non-bovine hosts. This finding suggests the possibility that deer carrying B. annulatus and moving across quarantine lines might, in addition to the tick, be carrying a cattle Babesia, which could prove highly pathogenic for U.S. cattle, and re-establish both disease and vector in the southern U.S. In addition to the obvious economic considerations for the livestock industry, it is probable that considerable ill will toward the maintenance of wildlife populations in these border areas would ensue.

This study was undertaken to determine the ability of the cattle Babesia, occurring in Mexico, to infect deer by placing infected B. annulatus larvae on them and allowing them to feed and by needle transfer of infected blood. In addition, trials, were conducted to determine the ability of infected ticks to transmit babesiosis to cattle after one generation on deer.

#### Materials and Methods:

In trial 1, deer 471 (figure 1) and splenectomized calf 187 were each

Personal Communications: W. J. Turner, Animal Health Division, Laredo, Texas, O. H. Graham, Entomology Research Division, Kerrville, Texas, and M. A. Price, Texas A&M University, College Station, Texas.

infested on day 0 with 1 g of B. annulatus larvae (1 g = 20,000 eggs) from a common pool, that had been obtained from engorged females that had fed on a Babesia infected calf native to Mexico. Blood samples were taken from each animal at weekly intervals beginning 2 weeks before tick infestation and continuing for at least 9 weeks after, except where death intervened. On day 7, 14, 21, 28, and 35, 10 ml of blood from deer 471 was collected in 0.5 ml of 12% sodium citrate and injected subcutaneously (s/c) into a susceptible splenectomized calf to check for evidence of a non-apparent latent or transient parasitemia. Blood samples from this calf were examined weekly for evidence of babesiosis. A large number of engorged females were harvested from deer 471 and allowed to oviposit. The resulting larvae were pooled, and 1 g placed on a susceptible splenectomized calf.

In trial 2, deer 124 (figure 2) and splenectomized calf 237 were each infested on day 0 with 2 g of B. annulatus larvae collected from a common pool, having been recovered from engorged females that had fed on a Babesia infected calf native to Mexico. Blood samples were taken at weekly intervals as described for trial 1. On days 7, 14, 21, 28, 35, and 42, 10 ml of blood from deer 124 was injected s/c into a susceptible splenectomized calf to check for evidence of latent Babesia infection. Weekly blood samples, from this calf, were examined for evidence of Babesia. A large number of engorged females were harvested from deer 124 and allowed to oviposit. The resulting larvae were pooled and when ready to feed, 2 g were placed on a susceptible splenectomized calf.

In trial 3, deer 475 and splenectomized calf 177 were each inoculated s/c on day 0 with 10 ml whole blood collected from a Babesia carrier calf. On days 7, 14, 21, 28, and 35, 10 ml of blood from deer 475 was injected

into a Babesia susceptible splenectomized calf. On day 58, deer 475 was splenectomized.

In trial 4, 28 days after splenectomy, deer 475 and splenectomized calf 179 were each inoculated s/c with 10 ml whole blood collected from a Babesia carrier calf. On days 7, 14, 21, and 28, 10 ml of blood from deer 475 was injected into a Babesia susceptible, splenectomized calf.

During all experiments blood samples were collected at weekly intervals, and daily observations made for signs of illness. Packed cell volume (PCV) determinations and Giemsa-stained blood smears were examined for evidence of Babesia infection. Rectal temperatures were taken of all calves exhibiting signs of clinical illness.

Babesiosis, as indicated in the results, was diagnosed on the basis of a Babesia parasitemia associated with a temperature rise, anemia, and hemoglobinuria. The babesiosis encountered in Mexican cattle used in these experiments appeared to be a mixed infection of B. bigemina and B. argentina.

Results:

Calf 187 (figure 1) died of acute babesiosis 16 days after being exposed to B. annulatus larvae. Larvae from the same source failed to produce evidence of Babesia infection in deer 471. A susceptible splenectomized calf failed to show evidence of Babesia infection after having been inoculated with blood from deer 471; 7, 14, 21, 28, and 35 days after tick release. A second generation of larvae from deer 471 attached and readily fed on a splenectomized calf but failed to transmit a Babesia infection.

Calf 237 (figure 2) died of acute babesiosis 13 days after being exposed to 2 g of B. annulatus larvae. Larvae from the same source failed

to show evidence of Babesia infection after having been inoculated with blood from deer 124; 7, 14, 21, 28, 35, and 42 days after tick release. A second generation of larvae from deer 124 attached and readily fed on a splenectomized calf but failed to transmit Babesia infection.

Calf 177 died of acute babesiosis on day 9 after having been injected s/c with 10 ml of blood from a carrier calf. Deer 475 after receiving a similar exposure failed to develop signs of Babesia infection. Attempts to recover Babesia from deer 475 by sub-inoculations into a splenectomized calf on days 7, 14, 21, 28, and 35 were negative. There was no evidence of a developing infection after the splenectomy of deer 475.

Calf 179 died of acute babesiosis on day 12 after having been injected s/c with 10 ml of blood from a carrier calf. Splenectomized deer 475 after receiving a similar exposure failed to develop signs of Babesia infection. Attempts to recover Babesia from deer 475 by sub-inoculations into a splenectomized calf on days 7, 14, 21, and 28 were negative.

#### Discussion and Conclusions:

It would be premature to state that white-tailed deer cannot be infected with cattle Babesia, but these experiments clearly indicate that deer do not develop a persistent, detectable, parasitemia following exposure to known infected ticks or by the inoculation of known infected blood from sources available to us. Ticks carrying Babesia were in both instances unable to transmit Babesia infection after having undergone a life cycle on white-tailed deer.

If the pattern of Babesia transmission, observed in these experiments, also occurs under natural conditions, it can be expected that ticks dropping off white-tailed deer are no longer capable of transmitting babesiosis. This does not mean that tick infested deer crossing the border are not capable of re-introducing B. annulatus into the border areas, but

perhaps in so doing they do not re-introduce babesiosis. Subsequent tick generations in contact with Babesia carrier cattle whether in the U.S. or Mexico could be expected to become infected and hence transmit the disease.

Abstract:

Attempts to induce a demonstrable cattle Babesia infection by feeding known infected ticks on two white-tailed deer were unsuccessful. The injection of known Babesia carrier blood into an intact and a splenectomized deer failed to result in evidence of infection.

All deer were checked for possible sub-patent infections by inoculating their blood into splenectomized calves at weekly intervals for 5 weeks following exposure, but no infections were produced in the calves.

Babesia infected ticks having undergone one generation on deer were unable to transmit infection to splenectomized calves on the succeeding generation.

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Figure 1  
Deer 471

Attempted Tick Transmission Of Babesia

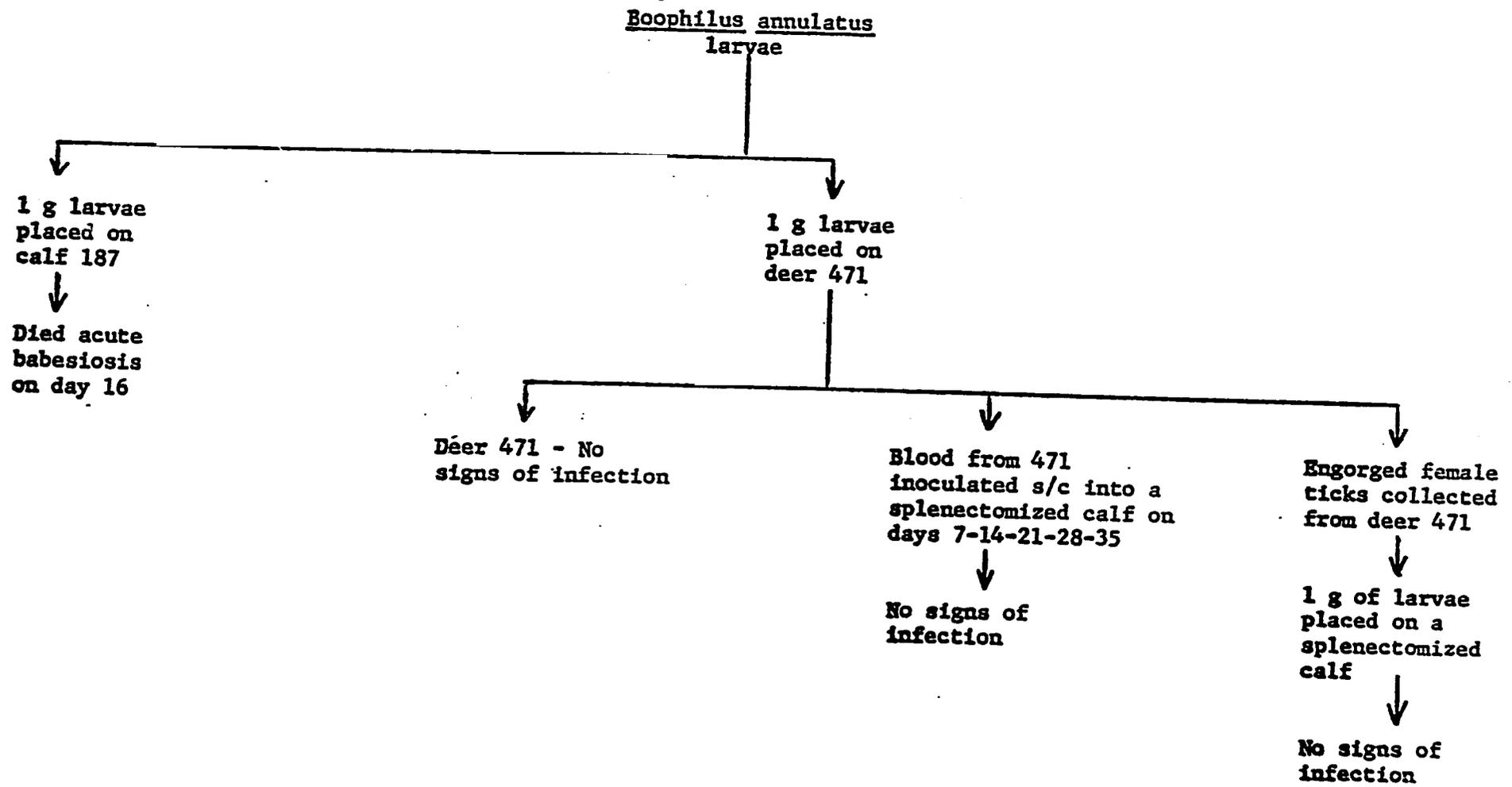
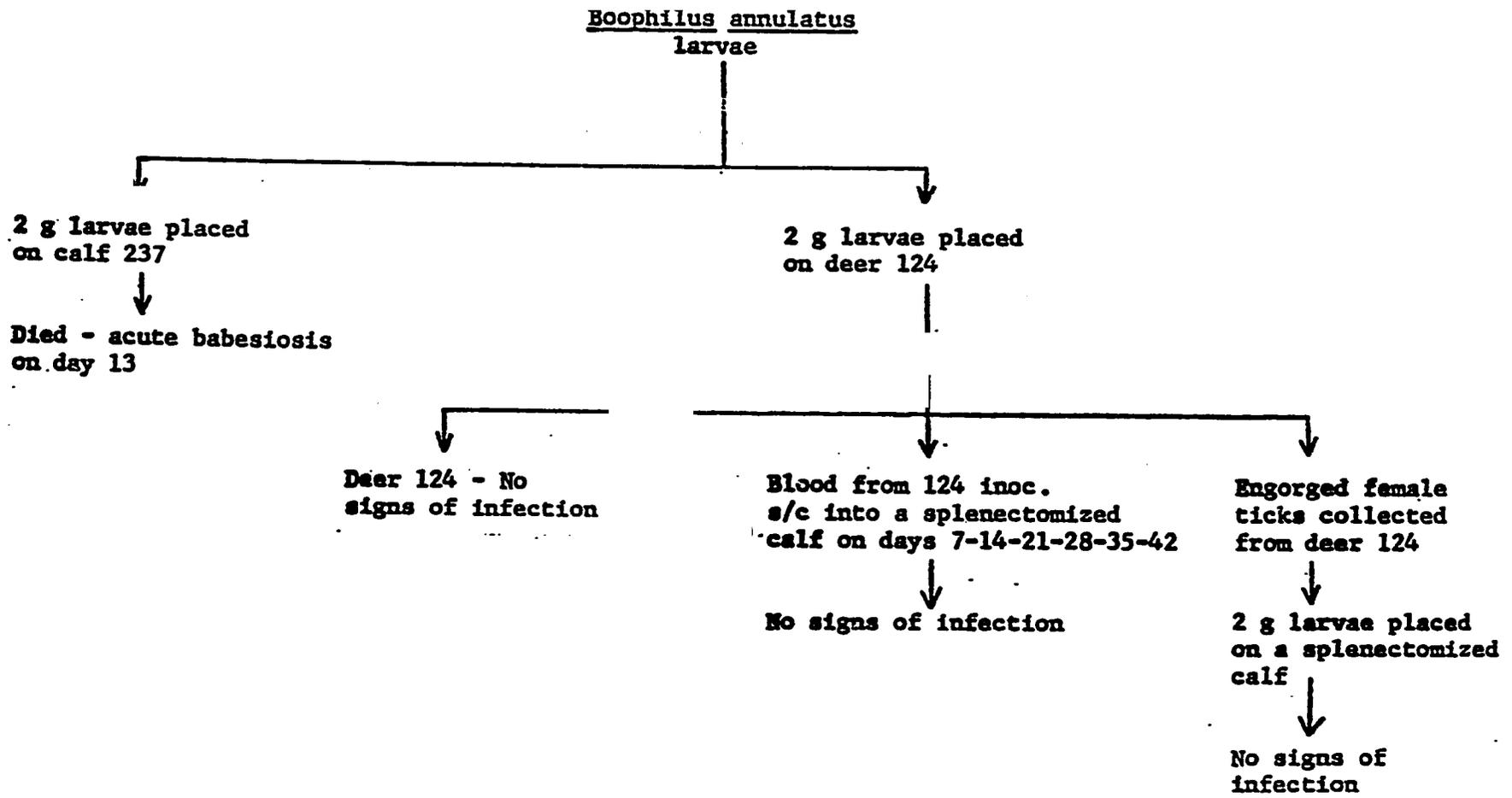


Figure 2  
Deer 124

Attempted Tick Transmission of Babesia



### Influence of Heat Stress on Milk Production

The conversion of forage and grain to milk and beef by the ruminant presents a potential which could significantly alter the supply of high quality protein to human diets, and provide a more nutritious diet to millions now living in tropical areas on minimum food intake. Frequently feed for cattle is abundant in these areas, but because of a combination of factors productivity is sometimes low. Some of the recognized factors are:

1. Genetic characteristics of local, indigenous cattle.
2. Climatic factors, which create stress factors, thus retarding productivity.
3. General management procedures.
4. Animal diseases including:
  - a. External parasites.
  - b. Internal parasites.
  - c. Hemo-parasites.
  - d. Bacterial and viral diseases.

An interrelationship of these factors is recognized, but probably not fully understood. The introduction of high producing European type dairy and beef cattle into many of these tropical areas is often followed by a loss in productivity assumed to be due primarily to climatic stress, and disease factors. Local cattle, which become adapted to the local conditions are frequently economically unproductive insofar as milk is concerned. Cross breeding offers the hope of improved vigor and productivity, but a drop in milk production generally follows the dilution of dairy types with beef characteristics.

There is probably room for improved management procedures to include loafing sheds, air conditioning, control of ticks by regular spraying, improved forage pastures and grain production.

Improved management, will contribute to disease control, but the complete control of these problems must yet be established through the use of vaccines and therapeutic agents that are yet to be developed by research and field application of techniques developed in the laboratory.

High milk production is readily achieved at those altitudes where temperatures are moderate, and where parasitic disease factors are minimized. This is dramatically illustrated when the dairy operations at Turipana and Tibaitata are compared.

A cursory examination of dairy productivity in Texas (College Station) reveals some common factors which might contribute to our understanding of the problems of milk production in the tropics. During the summer months the temperatures occurring in College Station closely resemble those occurring at Turipana (Table 1). There appears to be no significant differences between temperatures occurring in June-July-and August, when the average maximums and minimums are considered. The diurnal temperature fluctuation is basically the same at both locations. There is a difference in relative humidity, which may be a factor in creating an increased stress situation in Turipana. The humidity figures obtained for Turipana were monthly averages, and it is not known if they are comparable to the averages obtained in Texas. The diurnal fluctuations in relative humidity is extreme in Texas. As an example in August (1969) the average high R.H. was  $98.7 \pm 1.8$ , whereas the average low was  $33.9 \pm 16.8$ . It is not known if the R.H. at Turipana was an average or a value for a particular time of day, or an average of the high and low, or a true average recorded by averaging values throughout the day.

Temperatures at College Station during December, January and February are not recorded, but are known to be comparatively cool.

Milk production has been recorded during the summer months at College Station, where temperatures were comparable to those at Turipana, but where the parasitic disease factors are not present, and where management was good. The results are summarized in Table 2.

This study involved 28 cows divided equally in 4 groups, including 2 breeds, Jerseys and Holsteins observed for milk production during 2 seasons, winter (December, January and February) and summer (June, July and August).

During the summer the average daily milk production, expressed as pounds, was recorded for each of 7 Holstein cows beginning in June, 61 ( $\pm$  25) days after parturition, and continuing for the months of July and August 1969. Similar values were obtained for 7 Holstein cows beginning in December, 73.4 ( $\pm$  17.6) days after parturition and continued in January and February. Production records were tabulated for 7 Jersey cows beginning in June, 75.6 ( $\pm$  28.5) days after parturition and continuing to July and August. Similar values were obtained for 7 Jersey cows beginning in December, 72.3 ( $\pm$  19.7) days after parturition, continuing through January and February. The average production for each group, was tabulated (Table 2) for the summer and winter months with an analysis of variance conducted on the winter and summer groups to detect possible significant differences in milk production.

There was an obvious month to month decline, reflecting the extension of the lactation period. A regression coefficient was calculated for each cow and these compared by groups to determine differences in the rate of diminished milk production in each of the 4 groups. Statistical analysis was done on the regression coefficients to determine if heat stress or breed influenced a greater or lesser drop in production.

The results show no significant alteration in summer or winter milk

production among either the Jerseys or Holsteins. The regression coefficient, tabulating the rate of decrease, proved to actually be less in Jerseys during the summer than the winter months, which is opposite the expected results. Among Holstein cows the rate of reduction in milk production was greater during the summer months, when compared with Holstein production in the winter, but not significantly different. Comparisons between the regression coefficients of Holstein and Jersey in the summer, showed the Holstein rate of decrease to be significantly greater than seen in the Jersey.

Conclusion:

We were unable to demonstrate a climatic influence on milk production of Jersey and Holstein cows. These results suggest that the disease and management factors are probably items of major importance since cattle free of disease at Texas, stressed by the same temperatures and factors seen at Turipana failed to drop in production. More detailed studies are indicated, but it would appear from this preliminary investigation that high temperatures, and high humidity have been over emphasized, and that economical production is possible under tropical conditions if other inhibiting factors are controlled.

Comparative Temperatures and Humidity at  
College Station, Texas and Turipana, Colombia  
for the Months of June-July and August

	Texas - College Station 1969			Colombia - Turipana		
	June	July	Aug.	June	July	Aug.
Avg. Maximum Temperature	90.6°F ± 6.4	98.1°F ± 2.3	96.9°F ± 5.4	97.2°F	97.5°F	94.6°F
Avg. Minimum Temperature	71.5°F ± 6.9	76.3°F ± 2.1	75.2°F ± 2.1	68.0°F	77.7°F	67.1°F
Avg. Diurnal Temp. Fluxuation	19.1°F	21.8°F	21.7°F	29.2°F	19.8°F	27.5°F
Avg. Relative Humidity	68.5% ± 8.7	66.8% ± 7.8	67.5% ± 12.0	87%	86%	88%

**Comparative Winter and Summer Milk Production in  
Jersey and Holstein Cows  
(1969-1970)**

	Holsteins			Jerseys		
	Summer*	Winter*	Sig.	Summer*	Winter*	Sig.
No. of Animals	7	7		7	7	
Avg. No. of Days Post Parturition	61.0	73.4	NS	75.6	72.3	NS
Avg. Milk Production in Pounds	55.3	53.43	NS	38.2	33.8	NS
Avg. Regression in Milk Production	-0.2738**	-0.1476	NS	-0.119**	-0.176	P<0.05

\*Summer: June-July and August (1969).  
Winter: December-January and February (1969-70).

\*\*These regression coefficients are significantly different with P<0.05.

### Calf Weights: Tape Estimates Compared to Actual Weights

Consideration has been given the accuracy of tape measure weight estimates, in view of plans to carry on a treatment program at Nuevo Laredo and the need to rely on these estimates. These tape measures are placed around the girth, just behind the front legs, and based on the circumference an estimate of weight is made. We have weighed 19 calves ranging in weights from 90 to 280 pounds, and then estimated weights with the tape measures. These calves were almost entirely dairy types, Jerseys and Holsteins. The average weight from scale measure was 158 pounds, whereas the average weight based on the tape measure estimates was 181. Using paired analysis of differences it was found that tape measure weight estimates were significantly ( $P < 0.01$ ) higher than actual weights. For this group of calves an 18.9% error was detected.

A regression analysis made on the percent error versus weight showed a decreasing percent error in heavier animals. By projecting the estimates of error based on the regression coefficient it is estimated that animals weighing 100 pounds have a percent error of 23.3% whereas calves weighing 300 pounds will show an estimated error of only 7.9%, when actual weights are compared to tape measurements. The tape measurements for estimating weights can be expected to be more accurate in heavier animals.

### Future Research

In general the program at Texas A&M will be oriented toward the completion of present projects and utilization of past research results to evolve systems and techniques for the more efficient and economic control of hemotropic diseases of food producing ungulates.

Research at Texas A&M in the past has been productive, even though handicapped to some extent, in that field studies on all agents are impossible. Even though the pathogenic cattle Babesias and T. vivax are not present in Texas, studies with Babesia, trypanosomes, and Anaplasma are in progress. In addition, studies are being conducted on a Theileria, not known to be present in South America, but which is morphologically similar to the causative agent of East Coast Fever, occurring in East Africa. Research emphasis is being placed on anaplasmosis, largely because it is a local problem, field studies are possible, and the agent is readily available.

#### Anaplasmosis:

1. Recent advances, as reported in our annual reviews, in the treatment of anaplasmosis have encouraged continued effort in the field. Preliminary results show a new drug 4A65 (Imidocarb) to be an effective treatment. Studies are currently in progress and will be continued to evaluate this drug not only as a treatment of the clinical disease, but as a sterilizing and prophylactic agent. The route, dose, and treatment interval are and will continue to be studied. Field studies are planned for 1972 to better evaluate techniques which appear to be useful.

2. The in vitro cultivation of Anaplasma has been an illusive goal for many investigators. To date no confirmed reports of successful cultivation have appeared in the scientific literature, but the continued progress in tissue culture techniques, offers hope for the future. The value of success in this endeavor is obvious to the worker in the field, and it is generally recognized that to accomplish this would represent a major breakthrough in our understanding of this agent. Numerous tissues, media, and conditions are being used with a manipulation of many variables in an effort to achieve success.
3. Vaccines, both killed and attenuated live cultures have been developed, but at the present time neither are perfected to the point that they are routinely used in the tropics. The killed vaccine has a serious drawback in that the contaminating red cell antigens appear responsible for a hemolytic disease of the new born calf. To avoid this problem, means of purifying the basic antigen, or the elimination of the contaminating red cell antigens are being studied. This study will continue, several approaches offer the possibility of success.  
  
As a part of this study and as an adjunct to tissue culture techniques, attempts are being made to prepare antigens in small volumes to quantitatively evaluate the presence or absence of antigens in various tissues, and to chart the relative infectivity of blood in the infected animal. This study will continue and will include serologic techniques other than complement-fixation to evaluate the antigens present from various sources.

The value, limitations, and characteristics of the attenuated vaccine are and will continue to be investigated. A means of better preservation is an essential part of this study and will be pursued in the future. The problems of reversion to virulence, while partially academic, must be more thoroughly evaluated. A source of antigen, or the infectious organism other than red cells is being sought. There is some indication that high titers occur at times in the plasma. This occurrence is however unpredictable at the present time with no means of collecting antigens from the plasma. Studies are in progress along this line which may well contribute to the storage problem, and the elimination of red cell antigens.

Babesiosis:

1. A drug screening program is in progress in cooperation with The Division of Medicinal Chemistry, Walter Reed Army Institute of Research. A rodent Babesia (B. rodhaini) is being used, and protocols have been developed for the statistical evaluation of new and experimental drugs that may have use as babesiacides. Using this approach we can screen reasonably large numbers of compounds for specific activity.
2. Studies of the therapeutic efficacy of 4A65 (Imidocarb) on bovine Babesia in the vertebrate and invertebrate hosts will be conducted in cooperation with The Entomology Research Division, USDA, at their Nuevo Laredo facility. This program will be partially financed by a grant in the amount of \$2,500.00 from the Burroughs-Wellcome Research Foundation. It will also be determined if the drug will influence the Boophilus, and its ability to re-infect clean calves. The drug effect on the existing

infection in both the calf and the tick will be measured.

3. The prophylactic effect of 4A65 on Babesia bigemina will be conducted at Texas A&M in controlled laboratory experiments. A known infective inoculum will be administered at various levels needed for the treatment of Babesia, will be compared to those required for the treatment of Anaplasma. This work is contingent upon receiving permission to work with bovine Babesia. Isolation facilities are available and a request has been made.

#### Trypanosoma:

Work in this area is limited to studies with T. theileri, the non-pathogenic agent occurring in Texas. Plans have been made to culture this organism on various artificial medias, including embryonated eggs, for the production of serologic antigens which may be useful in the diagnosis of trypanosome infections. It is anticipated that common antigens occur between this organism and other more pathogenic trypanosomes. In any event, work on this agent will provide a useful model for possible duplication with other trypanosome pathogens.

#### Theileria:

There are striking morphologic similarities between the Theileria as seen on Giemsa stains, and some types of Anaplasma when examined with new methylene blue. There is also some evidence that deer infected with Theileria give specific response to the Anaplasma CF test. A study is being planned to more thoroughly evaluate the serologic and possible immunologic similarities of these 2 organisms, by cross reactions with their antigens and cross infectivity trials in splenectomized deer. It is becoming increasingly clear that there are antigenic variants among the T. parva

organisms in East Africa. It is hoped that the model we have of T. cervi might contribute some technique, or insight into the immunization problems encountered with East Coast Fever.

Project Title: Anaplasmosis - Tissue Culture

Project Number: H1630

Principal Investigator: R. J. Hidalgo

During the past year the entire effort on the Anaplasmosis Project, has been directed toward propagation of Anaplasma marginale in cell culture. To date, both static and roller cultures of the following cells have been screened for this purpose: Human synovial and porcine kidney cell lines; primary cell cultures of whole mouse embryos and rabbit kidneys; primary bovine cell cultures including spleen, lymph node, hemolymph node, platelet and erythrocytes and continuous-passage cells derived from peripheral leucocytes. Bovine erythrocytes were maintained in culture over feeder cell layers of bovine lymph node, bovine hemolymph node and bovine leucocytes.

Washed erythrocytes taken from a splenectomized calf with 86 percent of its erythrocytes parasitized by A. marginale were stored in liquid nitrogen for use as inoculum for cell cultures. Infectivity of the inoculum was proven after four months of storage in liquid nitrogen by inoculation of a splenectomized calf. Cell cultures were inoculated with varying quantities of infected erythrocytes. After inoculation, cultures were incubated for periods of from 7-35 days.

Cultures were monitored during incubation for growth of A. marginale by microscopic examinations of coverslips from Leighton tubes prepared, inoculated and incubated in the same manner as other cultures. In the case of erythrocyte cultures, smears of the red cell suspensions were prepared. For microscopic examination, coverslips and smears were stained

by Giemsa and acridine orange and by the direct fluorescent antibody technique. After incubation, cultures were frozen, thawed and subjected to ultrasonic oscillation. Whole culture sonicates were then concentrated to half volume by centrifugation and were titrated for the presence of antigens of A. marginale by the complement-fixation microtechnique (MCF).

Results of MCF titrations indicated that static and especially roller cell cultures of bovine leucocytes, lymph node and erythrocytes over lymph node cell feeder layers showed the most promise for propagation of A. marginale. However, increases in titers of inoculated cultures over controls were slight. Therefore, these serologic data are considered equivocal.

Microscopic examination of preparations stained by the direct fluorescent antibody technique appears to be the method of choice for detection of growth of A. marginale. However, due to the small size of the organism and some autofluorescence of culture cells, improvement of this technique is indicated. Acridine orange stains are useful but are not specific for the organism. Giemsa stains have not proven to be of much use. Microscopic examination of coverslips and erythrocyte smears stained by the fluorescent antibody technique indicate evidence that the normal bovine erythrocyte maintained over feeder cell layers of bovine leucocytes and especially lymph node cells support multiplication of A. marginale.

Work currently in progress or that will begin shortly will include: use of inoculum directly from infected calves rather than frozen inoculum; alteration of the cell culture media by the addition of serums from various species, increased concentrations of certain vitamins, etc.; use of splenectomized calves for detection of growth of A. marginale in cell culture and improvement of in vitro methods of detecting growth of the organism. New cell types will be screened for propagation of this organism. However,

as a result of previous studies, use of bovine erythrocytes and lymph node cells will be emphasized.

**PROGRESS REPORT OF HEMOTROPIC DISEASE RESEARCH**

**APPENDIX V**

**May, 1972**

**College of Veterinary Medicine  
Texas A & M University  
Cali – Columbia – South America**

INSTITUTE OF TROPICAL VETERINARY MEDICINE

TEXAS A&M UNIVERSITY GROUP

IN COLOMBIA

MAY, 1972

STUDIES ON HEMOTROPIC DISEASES

PROGRESS REPORT

PREPARED BY L. G. ADAMS<sup>1</sup> and R. A. TODOROVIC

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#### ACKNOWLEDGEMENTS

The United States Agency for International Development, the Rockefeller Foundation, the Centro Internacional de Agricultura Tropical, the Instituto Colombiano Agropecuario and Texas A&M University are gratefully acknowledged for their generous financial, facility, and administrative support. Furthermore, we would like to express our gratitude to the many collaborating veterinarians and other personnel of the above mentioned institutions, agencies and experiment stations.

## FRONTAGE

This annual reports is intended to be a document of progress because many of the experiments are in the primary stages of development, and therefore, only preliminary data are available now. Final results will be given in the next annual report.

Reviews of literature and bibliographies were intentionally deleted from all the individual experimental project reports to shorten the report. The data included in this report are not to be published or otherwise communicated without the permission of the authors.

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A Study of the Use of An Integrated Control Program for Hemo, Endo and Ectoparasites of Cattle on the North Coast of Colombia.

INTRODUCTION

Production losses in cattle on the Northern Atlantic Coast of Colombia due to infection or infestation, as the case may be, of the combination of hemoparasites, endoparasites and ectoparasites are apparently quite high and even restrictive in many zones of this region of Colombia. Previous studies have disclosed that anaplasmosis, babesiosis, trypanosomiasis, helminthiasis, acaridiasis and other insects are parasitic menaces causing, as a combination, high production losses. The control systems which the cattleman are now using are not optimally effective in reducing these losses, therefore, a better, modernized, economical control system should be developed.

The experimental control systems, hereindescribed are designed to reduce these losses to a minimum with a reasonable investment of capital; for this reason, three control systems are described, each one is successively less intricate, and costly, and are compared with a non-treated group. Should any of the 3 control systems prove economically acceptable, they would be, recommended for use on a national scale. Apart from the above mentioned objectives, a better insight as to age-incidence-prevalence of these diseases can be accomplished for future application.

MATERIALS AND METHODS

Site                      Turipaná ICA experimental Farm located at Montería, Córdoba, 20 m above sea level and the new Laboratorio de Investigaciones Médicas Veterinarias.

## GROUP 1

## Optimal Control System of Hemo, Endo and Ectoparasites

Animals	Newborn calves of Costello con cuernos and Sanmartinero Cows.
Number	19 calves
Drugs	Ganaseg (Squibb) Terramycin (Pfizer) 356-C-61 (Burroughs-Wellcome) Dursban (Dow) Toxaphene (Cooper) Bovizole (Merck) i Phenothiazine (Cooper)
Dosage & Route	Ganaseg 1.5 mg/kg IM Terramycin - 12 mg/kg IV 356-C-61 -5 mg/kg IV Dursban -1:600 topical Toxaphene -1:500 topical Bovizole -110 mg/kg oral Phenothiazine -25 mg/kg oral
Frequency	Ganaseg -Once at 10 days PI Terramycin and 356-C-61 in saline -Once at 21 PI Dursban -Each 10 days until 60 days are completed, thereafter each 17 days. Also the insecticides were alternated each 90 days.

Bovizole or Phenothiazine -Each 34 days, alternating the anthelmintics, each 34 days.

Premunition Day 0 -5 cc of blood containing Turipanã isolates of A. marginale, B. bigemina and B. argentina SQ.

Day 10 -Treatment against babesiosis with Ganaseg

Day 21 -Treatment against anaplasmosis with oxytetracycline and 356-C-61 in saline.

Duration 308 days.

## GROUP II

Intermediate Control System of Hemo, Endo and Ectoparasites

Animals Same as Group I

Number 20 calves

Drugs 4A65 (Burroughs-Wellcome)

Dursban (Dow)

Toxaphene (Cooper)

Bovizole (Merck)

Phenothiazine (Cooper)

Dosage & 4A65 -2.5 mg/kg SQ

Route Dursban -1:600 topical

Toxaphene -1:500 topical

Bovizole -110 mg/kg Oral

Phenothiazine -25 mg/kg Oral

Frequency 4A65 -Day 0 and Day 45 PI

Dursban or Toxaphene -Each 17 Days, alternating the insecticides each 90 days.

Bovizole or Phenothiazine -Each 34 days, alternating  
the anthelmintic each 34 days

Duration 308 days

### GROUP III

#### Minimal Control System of Endo and Ectoparasites

Animals	Same as group I
Number	20 calves
Drugs	Dursban (Dow) Toxaphene (Cooper) Bovizole (Merck) Phenothiazine (Cooper)
Dosis &	Dursban -1:600 Topical
Route	Toxaphene -1:500 Topical Bovizole -110 mg/kg Oral Phenothiazine -25 mg/kg Oral
Frequency	Dursban or Toxaphene - Each 17 days, alternating the insecticides every 90 days. Bovizole or Phenothiazine -Each 34 days, alternating the anthelmintics every 28 days.
Duration	308 days.

### GROUP C

#### CONTROL

Animals	Same as Group I
Number	19 calves

Drugs	None
Dosis	None
Frequency	None
Duration	308 days

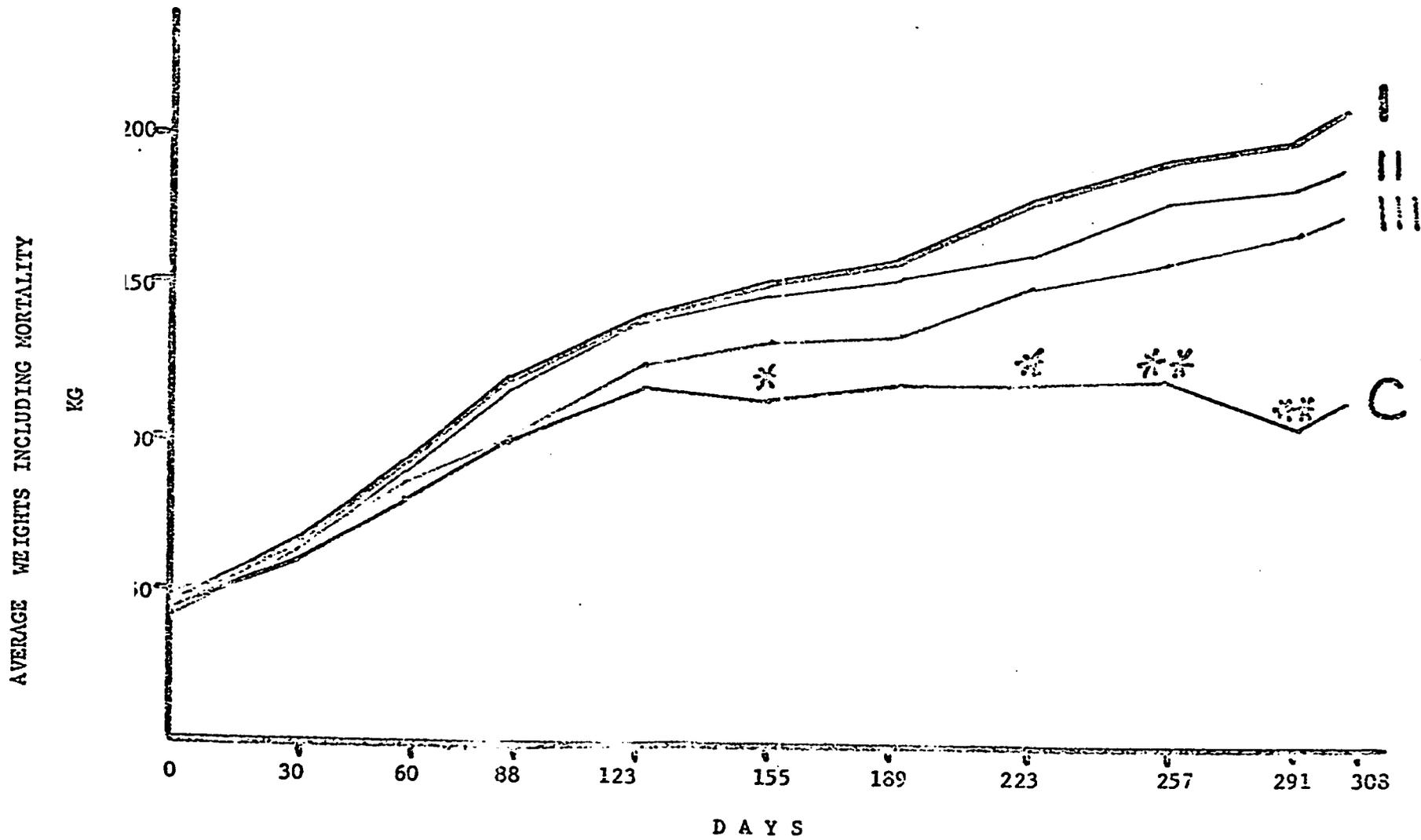
#### Parameters and Other Treatments

Packed cell volumes, blood smears, wet mounts, and complement fixation for anaplasmosis and babesiosis were determined every 10 days until the calves were 2 months old, and thereafter every 17 days. Body weights were determined every 34 days. Tick counts on all calves in each group were performed every 17 days beginning on day 155 PI. Helminth egg and larvae counts were done every 34 days. Temperatures will be taken during the premunition of Group I and when indicated in the other groups.

The mother cows of all the calves in the experiment were bled for parasitemias, PCV, wet mount and CF determinations. All calves were newborn, i.e., less than 10 days old when the experiment started. Day 0 is considered to be the day when the premunition procedure in Group I, the application of 4A65 in Group II and anthelmintics in Group III were started; the average of the calves was 30 days. An attempt was made to begin all treatments when the calves were 25 to 35 days of age. All cattle were vaccinated as indicated against blackleg, hemorrhagic septicemia, malignant edema, and Foot and Mouth disease. All groups were treated on days 73 and 87 against Dictyocaulus viviparus with Francozan (Cooper) and Luvorem (Squibb) at the recommended dosages.

#### RESULTS

The results are given on pages 6 through 29.



WEIGHTS INCLUDING MORTALITY.

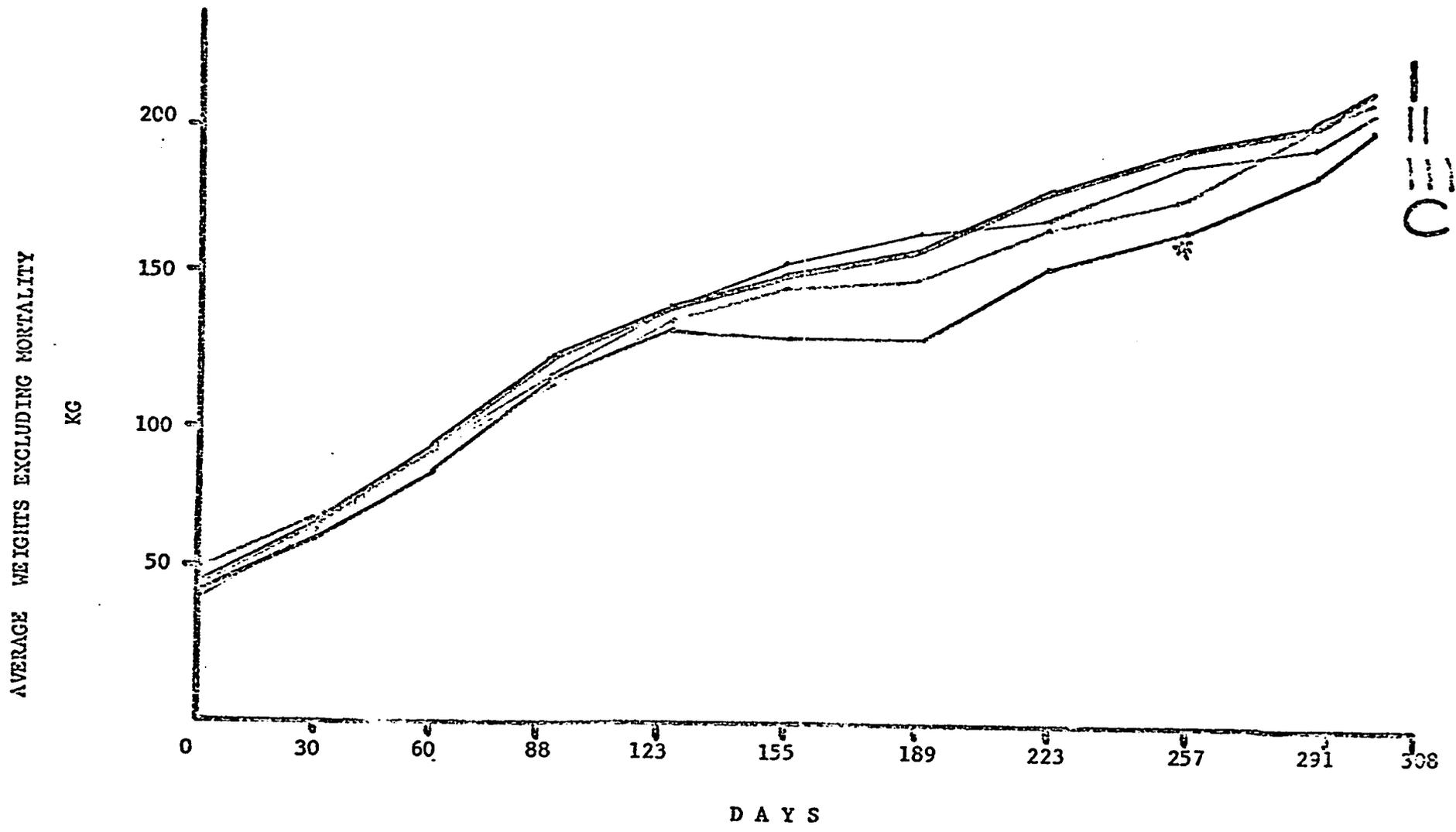
DAYS	GROUPS				
	I	II	III	CONTROL	F
	$\bar{x} \pm S_D$ kg	$\bar{x} \pm S_D$ kg	$\bar{x} \pm S_D$ kg	$\bar{x} \pm S_D$ kg	
0	46.21 $\pm$ 12.44	45.40 $\pm$ 8.69	48.85 $\pm$ 15.07	45.16 $\pm$ 8.66	0.425
30	70.53 $\pm$ 16.53	65.45 $\pm$ 12.76	66.65 $\pm$ 19.30	61.84 $\pm$ 13.77	0.823
60	93.53 $\pm$ 22.33	92.85 $\pm$ 18.81	85.50 $\pm$ 27.36	83.00 $\pm$ 20.44	1.09
88	119.37 $\pm$ 25.67	115.70 $\pm$ 15.19	99.95 $\pm$ 42.43	100.32 $\pm$ 42.44	1.80
123	137.95 $\pm$ 24.33	138.65 $\pm$ 17.83	125.85 $\pm$ 42.84	118.37 $\pm$ 47.47	1.51
155	149.58 $\pm$ 21.04	148.35 $\pm$ 40.35	133.50 $\pm$ 55.66	112.84 $\pm$ 54.84	2.74*
189	158.58 $\pm$ 22.11	153.70 $\pm$ 44.99	133.95 $\pm$ 59.02	120.32 $\pm$ 55.80	2.65
223	178.37 $\pm$ 25.15	161.65 $\pm$ 45.92	152.05 $\pm$ 66.39	122.11 $\pm$ 75.35	3.31*
257	192.58 $\pm$ 30.76	178.45 $\pm$ 28.62	159.75 $\pm$ 66.64	122.58 $\pm$ 83.72	4.72**
291	200.16 $\pm$ 26.14	183.10 $\pm$ 54.19	170.46 $\pm$ 81.50	106.75 $\pm$ 100.69	7.11**
308	209.80 $\pm$ 29.42	193.05 $\pm$ 55.25	176.23 $\pm$ 85.57	116.08 $\pm$ 112.74	2.43

AVERAGE DAILY GAINS INCLUDING MORTALITY

DAYS	GROUPS				
	I	II	III	CONTROL	F
	$\bar{x} \pm S_D$ grams	$\bar{x} \pm S_D$ grams	$\bar{x} \pm S_D$ grams	$\bar{x} \pm S_D$ grams	
0-257	569.53 ± 102.75	525.10 ± 161.53	450.59 ± 201.94	344.26 ± 400.19	5,20***

GROUP TOTAL WEIGHTS & ECONOMIC DIFFERENCES INCLUDING MORTALITY

	kg	kg	kg	kg
0-257 Loss	0	278	820	2,160
At 9.80/kg ADJUSTED DIFFERENCE	PESOS (US\$) \$21.168 (1,008)	PESOS (US\$) \$8.036 (383)	PESOS (US\$) \$2.724 (130)	PESOS (US\$) 0 0
- Treatment Cost	1.523 (73)	1.514 (72)	1.453 (2')	0 0
NET	\$19.645 (935)	\$6.522 (311)	\$1.271 (61)	0 0



WEIGHTS EXCLUDING MORTALITY

DAYS	GROUPS				
	I	II	III	CONTROL	F
	$\bar{x} \pm S_D$ kg	$\bar{x} \pm S_D$ kg	$\bar{x} \pm S_D$ kg	$\bar{x} \pm S_D$ kg	
0	46.21±12.44	45.40±8.69	48.85±15.07	45.16±8.66	0.425
30	70.53±16.52	65.45±12.76	66.65±19.30	61.84±13.77	0.823
60	93.53±22.33	92.85±18.81	90.00±19.52	83.00±20.44	1.129
88	119.37±25.67	115.70±15.19	111.06±27.13	112.12±25.69	0.488
123	137.95±24.33	138.65±17.83	134.47±32.40	132.29±24.81	0.357
155	149.58±21.04	156.16±21.15	148.33±33.35	134.00±25.04	2.310
189	158.58±22.11	161.79±28.30	148.83±39.18	134.47±39.47	2.565
223	178.37±25.15	170.16±26.23	168.94±42.45	154.67±43.63	1.288
257	192.58±30.76**	187.84±28.84**	177.50±39.52	166.36±42.81	11.010**
291	200.16±26.14	192.74±34.41	201.46±26.80	183.14±45.87	0.953
308	209.80±29.42	203.21±32.79	208.27±38.04	199.00±58.71	0.198

AVERAGE DAILY GAINS EXCLUDING MORTALITY

DAYS	GROUPS				
	I	II	III	CONTROL	F
	$\bar{x} \pm s_D$ grams	$\bar{x} \pm s_D$ grams	$\bar{x} \pm s_D$ grams	$\bar{x} \pm s_D$ grams	
0-257	569.53 $\pm$ 102.75	552.73 $\pm$ 106.86	500.66 $\pm$ 137.98	467.21 $\pm$ 169.21	2.20

GROUP TOTAL WEIGHT & ECONOMIC DIFFERENCES EXCLUDING MORTALITY

0-257 Loss	kg 0	kg 90	kg 464	kg 1330	
at 9.80/Kg Adjusted Difference	PESOS (US\$) \$13.034 (621)	PESOS (US\$) \$4.547 (217)	PESOS (US\$) \$882.00 (42)	PESOS (US\$) 0 0	
- Treatment Cost	1.523 (73)	1.514 (72)	1.453 (69)	0 0	
NET	\$11.511 (548)	3.033 (144)	-571 -- (27)	0 0	

MORTALITY

DAYS	GROUPS			
	I	II	III	CONTROL
	No.	No.	No.	No.
0	0	0	0	0
10	0	0	0	0
20	0	0	0	0
30	0	0	0	0
40	0	0	0	0
60	0	0	1 A	0
72	0	0	0	1A, 1A + EP
88	0	0	0	0
106	0	0	0	0
123	0	0	0	0
138	0	0	1A + B	0
155	0	1A	0	0
172	0	0	0	0
189	0	0	0	0
206	0	0	0	1A + VP + P
223	0	0	0	1VP
240	0	0	0	0
257	0	0	0	0
274	0	0	0	1A + VP
291	0	0	0	0
308	0	0	0	0
TOTAL	0	1	2	5
%	0	5	10	26.32

A = Anaplasma marginale  
 B = Babesia bigemina and/or Babesia argentina.  
 VP = Verminous Pneumonia - Dictyocaulus viviparus.  
 EP = Endoparasitism.  
 P = Pasteurellosis.

PACKED CELL VOLUME

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$
	%	%	%	%
0	34.2 $\pm$ 3.7	35.2 $\pm$ 6.0	33.8 $\pm$ 4.1	35.2 $\pm$ 3.8
10	30.5 $\pm$ 5.1	36.4 $\pm$ 5.7	33.8 $\pm$ 4.9	34.5 $\pm$ 4.1
20	35.1 $\pm$ 7.9	34.6 $\pm$ 5.6	30.6 $\pm$ 5.4	33.3 $\pm$ 5.4
30	32.1 $\pm$ 4.5	34.4 $\pm$ 5.6	30.8 $\pm$ 5.8	29.3 $\pm$ 4.4
40	33.3 $\pm$ 4.0	32.0 $\pm$ 7.8	31.8 $\pm$ 5.6	29.2 $\pm$ 4.7
60	31.9 $\pm$ 4.0	33.4 $\pm$ 4.8	31.2 $\pm$ 5.1	27.2 $\pm$ 6.0
72	31.4 $\pm$ 3.7	33.9 $\pm$ 4.9	30.8 $\pm$ 5.0	29.6 $\pm$ 5.0
88	29.6 $\pm$ 4.8	29.3 $\pm$ 4.9	29.7 $\pm$ 3.9	28.8 $\pm$ 4.0
106	29.4 $\pm$ 5.6	28.3 $\pm$ 6.4	29.9 $\pm$ 5.3	30.8 $\pm$ 4.4
123	30.8 $\pm$ 5.5	28.1 $\pm$ 7.0	27.9 $\pm$ 4.9	28.4 $\pm$ 4.3
138	27.9 $\pm$ 3.9	29.4 $\pm$ 6.5	28.1 $\pm$ 3.1	30.4 $\pm$ 5.7
155	26.2 $\pm$ 3.9	24.9 $\pm$ 4.8	29.9 $\pm$ 3.7	28.6 $\pm$ 3.4
172	25.9 $\pm$ 3.2	25.3 $\pm$ 4.5	28.6 $\pm$ 4.1	27.2 $\pm$ 3.2
189	26.7 $\pm$ 3.1	27.1 $\pm$ 5.4	29.7 $\pm$ 4.4	28.7 $\pm$ 3.4
206	25.6 $\pm$ 3.2	26.5 $\pm$ 3.3	29.9 $\pm$ 4.0	30.0 $\pm$ 5.0
223	27.7 $\pm$ 3.5	29.5 $\pm$ 2.8	30.5 $\pm$ 4.1	28.7 $\pm$ 3.3
240	29.7 $\pm$ 3.3	31.1 $\pm$ 2.6	31.5 $\pm$ 3.0	29.4 $\pm$ 4.1
257	30.4 $\pm$ 3.8	31.3 $\pm$ 3.0	31.4 $\pm$ 2.9	28.8 $\pm$ 4.3
274	32.4 $\pm$ 3.4	30.8 $\pm$ 4.3	31.0 $\pm$ 3.8	29.2 $\pm$ 3.5
291	31.2 $\pm$ 4.3	31.5 $\pm$ 3.0	30.8 $\pm$ 4.8	28.4 $\pm$ 2.3
308	29.5 $\pm$ 3.5	29.1 $\pm$ 3.1	32.3 $\pm$ 4.7	28.7 $\pm$ 3.0

ANAPLASMA MARGINALE PARASITEMIA

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$
	%	%	%	%
0	0	0	0	0
10	0	0.005 $\pm$ 0.022	0.003 $\pm$ 0.010	0.010 $\pm$ 0.036
20	0.813 $\pm$ 0.712	0.025 $\pm$ 0.045	0.045 $\pm$ 0.150	0
30	0.007 $\pm$ 0.017	0.026 $\pm$ 0.045	0.003 $\pm$ 0.010	0
40	0.047 $\pm$ 0.051	0.418 $\pm$ 0.783	0.053 $\pm$ 0.157	0.082 $\pm$ 0.353
60	0.042 $\pm$ 0.155	0.118 $\pm$ 0.278	0.147 $\pm$ 0.255	0.072 $\pm$ 0.543
72	0.321 $\pm$ 0.767	0.418 $\pm$ 0.783	0.211 $\pm$ 0.314	0.481 $\pm$ 0.226
88	0.137 $\pm$ 0.572	0.108 $\pm$ 0.205	0.279 $\pm$ 0.450	0.327 $\pm$ 0.753
106	0.316 $\pm$ 0.821	0.078 $\pm$ 0.236	0.332 $\pm$ 0.647	0.071 $\pm$ 0.188
123	0.171 $\pm$ 0.395	0.080 $\pm$ 0.206	0.332 $\pm$ 0.494	0.229 $\pm$ 0.458
138	0.255 $\pm$ 0.935	0.285 $\pm$ 0.797	0.183 $\pm$ 0.391	0.045 $\pm$ 0.121
155	0.290 $\pm$ 0.576	0.727 $\pm$ 1.151	0.418 $\pm$ 1.005	0.240 $\pm$ 0.766
172	0.447 $\pm$ 0.624	0.263 $\pm$ 0.362	0.030 $\pm$ 0.085	0.081 $\pm$ 0.108
189	0.724 $\pm$ 0.980	0.500 $\pm$ 0.627	0.142 $\pm$ 0.397	0.351 $\pm$ 1.240
206	0.397 $\pm$ 1.165	0.176 $\pm$ 0.255	0.341 $\pm$ 0.579	0.006 $\pm$ 0.014
223	0.129 $\pm$ 0.304	0.022 $\pm$ 0.062	0.008 $\pm$ 0.026	0.002 $\pm$ 0.037
240	0.045 $\pm$ 0.133	0.046 $\pm$ 0.113	0.009 $\pm$ 0.010	0.225 $\pm$ 0.829
257	0.011 $\pm$ 0.024	0.058 $\pm$ 0.172	0.031 $\pm$ 0.082	0.029 $\pm$ 0.111
274	0.006 $\pm$ 0.014	0.001 $\pm$ 0.000	0.028 $\pm$ 0.041	0.008 $\pm$ 0.026
291	0.028 $\pm$ 0.117	0.010 $\pm$ 0.020	0.026 $\pm$ 0.047	0.026 $\pm$ 0.040
308	0	0.002 $\pm$ 0.010	0.006 $\pm$ 0.010	0.021 $\pm$ 0.026

BADESIA BIGEMINA PARASITEMIA

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$
	%	%	%	%
0	0	0.050 $\pm$ 0.229	0	0.001 $\pm$ 0.000
10	0.076 $\pm$ 0.289	0	0	0
20	0	0	0	0.021 $\pm$ 0.073
30	0	0	0	0.026 $\pm$ 0.075
40	0	0	0	0
60	0	0	0	0
72	0	0	0	0
88	0.005 $\pm$ 0.022	0	0	0
106	0	0	0.003 $\pm$ 0.010	0
123	0	0	0.005 $\pm$ 0.016	0
138	0	0	0	0
155	0	0.008 $\pm$ 0.020	0.003 $\pm$ 0.014	0
172	0	0.002 $\pm$ 0.010	0.003 $\pm$ 0.014	0
189	0	0	0.003 $\pm$ 0.014	0.006 $\pm$ 0.024
206	0	0	0.013 $\pm$ 0.049	0
223	0	0	0	0
240	0.001 $\pm$ 0.000	0	0.002 $\pm$ 0.010	0
257	0.001 $\pm$ 0.000	0	0	0.004 $\pm$ 0.014
274	0.004 $\pm$ 0.010	0.003 $\pm$ 0.010	0	0.001 $\pm$ 0.000
291	0.002 $\pm$ 0.010	0.001 $\pm$ 0.000	0.003 $\pm$ 0.010	0.001 $\pm$ 0.000
308	0.006 $\pm$ 0.014	0.002 $\pm$ 0.000	0.003 $\pm$ 0.010	0.004 $\pm$ 0.010

BADESIA ARGENTINA PARASITEMIA

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$	$\bar{x} \pm S_D$
	%	%	%	%
0	0	0	0	0
10	0.005 $\pm$ 0.024	0	0	0
20	0	0	0	0
30	0	0	0	0
40	0	0	0	0
60	0	0	0	0
72	0	0	0	0
88	0.003 $\pm$ 0.010	0	0.079 $\pm$ 0.354	0
106	0	0	0.079 $\pm$ 0.340	0
123	0	0	0	0
138	0	0	0	0
155	0	0	0.001 $\pm$ 0.000	0
172	0	0	0	0
189	0	0	0	0
206	0	0	0	0
223	0	0	0	0
240	0	0	0	0
257	0	0	0	0
274	0	0	0	0
291	0	0	0	0
308	0	0	0	0

RECIPROCAL CF TITERS FOR ANAPLASMA MARGINALE

DAYS	GROUPS			
	I $\bar{x}$	II $\bar{x}$	III $\bar{x}$	CONTROL $\bar{x}$
0	0	1.6	1.2	0
10	1.6	1.2	1.2	1.2
20	1.4	2.4	1.5	0
30	10.0	3.1	1.5	1.4
40	2.9	8.1	2.1	1.5
60	1.4	2.7	4.8	6.8
72	2.5	5.4	3.2	1.3
88	2.7	4.4	5.0	8.8
106	2.8	5.3	10.0	5.2
123	3.1	2.7	5.5	11.0
138	3.3	6.4	4.8	6.1
155	3.0	5.9	5.8	5.3
172	6.1	3.7	6.4	5.9
189	2.4	2.4	3.5	2.4
206	4.9	3.6	5.0	3.1
223	3.6	2.6	4.1	3.9
240	3.7	2.7	4.6	4.1
257	2.5	3.2	4.3	2.1
274	3.1	2.2	5.5	2.3
291	3.2	3.7	6.7	2.5
308	4.0	3.2	5.4	1.6

RECIPROCAL CF TITERS FOR BABESIA BIGEMINA

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
0	1.6	1.3	1.4	1.7
10	1.6	1.2	3.7	1.4
20	2.2	1.2	1.2	1.4
30	1.6	1.2	2.9	6.1
40	1.1	0	3.2	5.7
60	5.6	0	3.8	5.0
72	4.6	0	1.5	4.2
88	7.8	0	4.8	5.4
106	1.5	0	7.9	5.7
123	4.9	1.1	6.4	3.6
138	3.9	1.3	3.1	4.5
155	5.7	6.8	5.5	4.7
172	5.2	6.4	3.6	4.9
189	2.1	3.2	4.0	4.4
206	3.0	4.4	1.8	3.5
223	4.3	5.0	3.7	5.3
240	4.3	4.0	5.2	4.8
257	3.4	3.2	3.9	2.9
274	3.0	3.9	4.5	2.8
291	2.8	3.1	4.2	3.1
308	3.2	4.0	3.9	2.5

RECIPROCAL CF TITERS FOR BABESIA ARGENTINA

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
0	0	1.2	1.2	0
10	0	0	0	1.2
20	0	0	1.2	1.5
30	1.2	0	1.2	1.5
40	1.9	0	1.7	1.1
60	3.3	0	1.1	1.8
72	1.3	0	1.4	1.6
88	2.4	0	1.9	1.2
106	3.6	1.2	2.0	2.1
123	3.0	1.4	1.5	2.8
138	1.3	1.7	1.4	1.6
155	1.5	2.6	1.3	1.7
172	1.3	2.6	1.3	1.7
189	0	1.4	1.3	1.4
206	1.6	2.0	1.4	1.8
223	1.4	1.5	1.5	0
240	1.4	1.3	1.1	1.4
257	0	1.6	1.4	0
274	1.9	1.0	1.2	1.5
291	1.4	1.8	1.8	0
308	1.4	1.1	2.9	0

INGUINAL TICK COUNTS

DAYS	GROUPS			
	.I	.II	.III	.CONTROL
	$\bar{x} \pm s_D$	$\bar{x} \pm s_D$	$\bar{x} \pm s_D$	$\bar{x} \pm s_D$
155*	65.7 $\pm$ 70.4	25.6 $\pm$ 29.8	10.5 $\pm$ 12.0	10.0 $\pm$ 9.5
172	22.5 $\pm$ 50.0	28.3 $\pm$ 31.4	9.7 $\pm$ 16.0	10.0 $\pm$ 16.4
189	9.9 $\pm$ 14.6	6.4 $\pm$ 7.6	19.4 $\pm$ 12.4	20.4 $\pm$ 25.8
206	10.4 $\pm$ 11.2	14.9 $\pm$ 15.3	12.4 $\pm$ 10.5	6.8 $\pm$ 5.8
223	5.7 $\pm$ 3.0	8.4 $\pm$ 5.6	19.8 $\pm$ 15.5	26.2 $\pm$ 16.0
240	14.0 $\pm$ 15.8	16.3 $\pm$ 33.0	14.4 $\pm$ 16.2	166.1 $\pm$ 192.1
257	20.6 $\pm$ 14.9	6.2 $\pm$ 22.7	21.2 $\pm$ 31.5	29.4 $\pm$ 41.2
274	8.8 $\pm$ 10.0	6.1 $\pm$ 8.3	35.1 $\pm$ 37.6	38.8 $\pm$ 27.9
291	1.3 $\pm$ 2.5	7.2 $\pm$ 13.2	8.4 $\pm$ 6.9	35.1 $\pm$ 26.4
308	1.8 $\pm$ 2.1	4.8 $\pm$ 4.7	15.8 $\pm$ 19.3	19.7 $\pm$ 11.3

\* From day 0 through day 138, all calves were infested with low levels of ticks that were below levels that could be efficiently counted.

BUNOSTOMUM SPP.

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
	eggs/g	eggs/g	eggs/g	eggs/g
0	0.00	0.00	0.00	0.00
30	0.32	0.10	0.00	0.00
60	0.00	0.00	0.00	3.00
88	0.26	0.00	0.41	2.30
123	0.11	0.00	0.79	3.30
155	1.79	0.11	1.00	2.00
189	0.42	0.11	0.17	7.06
223	0.28	0.95	0.94	11.79
257	0.22	0.32	0.06	3.38
291	0.05	0.00	0.00	7.57

COOPERIA SPP.

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
	eggs/g	eggs/g	eggs/g	eggs/g
0	0.00	0.00	0.10	0.00
30	0.00	0.00	22.50	0.58
60	0.00	0.15	8.42	4.47
88	3.32	2.26	15.24	13.88
123	4.16	18.50	71.95	61.44
155	21.16	22.47	50.33	81.88
189	99.53	30.26	35.56	71.94
223	18.78	100.21	57.12	5.53
257	2.16	20.58	53.89	1.46
291	0.95	5.89	0.73	20.43

DICTYOCAULUS VIVIPARUS

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
	larvae/g	larvae/g	larvae/g	larvae/g
0	2.42	1.50	3.15	2.16
30	8.42	2.75	0.95	0.26
60	1.32	1.50	0.53	1.32
88	0.00	0.05	0.00	0.05
123	0.00	0.00	0.11	0.06
155	0.05	0.00	0.17	0.24
189	0.16	0.21	0.25	0.59
223	0.50	0.11	0.13	3.67
257	0.06	0.32	0.22	1.38
291	0.00	0.22	0.00	0.00

EIMERIA SPP.

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
	eggs/g	eggs/g	eggs/g	eggs/g
0	631.68	73.70	6.25	77.05
30	31.58	69.50	441.80	344.84
60	72.79	20.80	143.65	158.11
88	230.42	38.42	271.56	280.82
123	71.53	251.35	172.84	22.88
155	268.11	329.50	329.56	25.82
189	422.43	384.68	171.39	86.88
223	166.17	201.26	145.56	17.40
257	74.50	13.16	88.06	6.08
291	23.05	3.21	0.82	16.71

HAEMONCHUS SPP.

DAYS	GROUPS			
	I	II	.III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
	eggs/g	eggs/g	eggs/g	eggs/g
0	1.95	0.00	0.00	0.00
30	3.68	0.20	0.00	16.47
60	0.00	0.00	0.00	0.11
88	0.00	0.00	0.11	0.74
123	0.37	0.80	0.21	5.83
155	0.63	3.40	2.22	2.94
189	1.89	0.06	1.72	10.29
223	1.39	0.32	0.59	6.87
257	0.72	0.32	0.06	0.69
291	1.58	0.74	0.00	1.43

MONIEZIA SPP

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
	eggs/g	eggs/g	eggs/g	eggs/g
0	0.00	0.00	0.00	1.05
30	0.00	0.20	0.00	0.00
60	0.00	0.00	0.00	0.00
88	0.00	0.00	38.82	1.05
123	0.00	0.00	0.00	8.42
155	0.00	0.00	0.00	10.00
189	3.95	4.11	0.00	0.00
223	5.68	0.00	0.00	0.00
257	0.00	0.00	0.00	0.00
291	1.42	0.00	0.00	0.00

OESOPHAGOSTOMUM RADIATUM

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
	eggs/g	eggs/g	eggs/g	eggs/g
0	0.00	1.55	0.00	0.00
30	0.00	0.00	0.00	0.05
60	0.00	0.00	0.21	0.74
88	0.00	0.11	0.35	0.37
123	0.16	3.55	0.00	0.00
155	0.00	0.95	1.67	2.06
189	4.16	0.00	1.00	3.53
223	0.00	1.58	0.24	0.87
257	0.00	0.79	0.56	0.69
291	0.00	0.21	0.00	0.29

STRONGYLOIDES SPP.

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
	Larvae/g	Larvae/g	Larvae/g	Larvae/g
0	87.42	104.20	623.50	881.21
30	0.11	20.10	71.50	521.95
60	220.16	269.90	21.32	401.11
88	4.68	2.84	22.06	85.06
123	1.21	34.16	23.89	31.88
155	11.89	15.26	66.94	20.00
189	28.05	28.11	15.61	25.00
223	27.68	52.89	27.53	1.40
257	1.67	13.53	6.33	0.00
291	1.47	0.32	0.18	2.86

TRICHOSTRONGYLUS SPP

DAYS	GROUPS			
	I	II	III	CONTROL
	$\bar{x}$	$\bar{x}$	$\bar{x}$	$\bar{x}$
	eggs/g	eggs/g	eggs/g	eggs/g
0	0.00	0.70	0.50	0.21
30	0.00	0.70	13.50	0.00
60	7.89	0.30	0.00	0.00
88	0.26	1.16	7.29	0.29
123	0.79	0.00	0.05	2.35
155	0.00	0.00	0.06	0.94
189	0.26	0.56	1.89	23.65
223	0.56	2.74	0.24	2.07
257	2.06	0.42	0.00	0.38
291	1.32	9.68	0.09	7.43

## DISCUSSION AND CONCLUSION

In reference to the endoparasite control program, the data (pg. 21-29) indicates that the application of anthelmintics generally reduced the endoparasite loads of Groups I, II and III as compared to the control Group C. However, the anthelmintics had little effect on Eimeria spp., Moniezia spp. or Strongyloides spp. Dictyocaulus viviparus caused the most significant loss in production and the highest mortality rate, and therefore, had to be treated in all groups on days 73 and 87 to eliminate interference with the hemoparasite treatments. If mortality was included, the anthelmintic treatments yielded increased weight gains (278 kg more than the control) and increased net monetary returns (US\$61 more than Group C), however, when mortality was not included the anthelmintic treatments yielded only 90 kg more than the controls and a net loss of US\$27. The most important effect of the endoparasite treatment was to reduce and control the level of endoparasites so that the hemoparasites could be better evaluated.

Because the ectoparasite control was applied at 10 day intervals for the first 60 days and at 17 day intervals, thereafter, tick counts were deemed unnecessary since the tick loads were very low until day 155. From day 155 through day 308, the tick counts (pg. 20) demonstrated that the insecticides had a variable control effects, e.g. the control group had higher numbers of ticks from day 223 through 308 at which the tick population of the pastures rose greatly. The insecticide control of ticks aided to reduce the amount of hemoparasite-tick field challenge in Groups I, II and III.

Groups III and C generally had packed cell volumes lower than or equal to Groups I and II, however, no significant differences occurred between the 4 groups. Groups III and C's packed cell volumes never decreased significantly

below Groups I and II which indicated that the hemoparasite challenge was not as great as was anticipated. The parasitemias (Pg. 14, 15, 16) of A. marginale, B. bigemina and B. argentina were correlated and consistent with the changes in the packed cell volumes. Group II, treated with 4A65, had an increased parasitemia of A. marginale, but no parasitemias of B. bigemina occurred until day 155 and B. argentina never appeared. The complement fixation titers (17, 18, 19) for A. marginale, B. bigemina and B. argentina were correlated and consistent with the parasitemias.

The mortality rates (Pg. 11) of Group C was very high due to anaplasmosis complexed with verminous pneumonia and internal parasitism, however, the primary cause of death in all calves except one in Group C was due to acute exacerbations of chronic anaplasmosis.

Average weight gains (Pg. 9, 10) and average daily weight gains (pg. 11) excluding mortalities were highest in Groups I and II throughout the experiment. On day 257, the average weights of Groups I and II were significantly ( $P < 0.01$ ) higher than the Group C, however, the average daily gains were not significantly different. If the mortalities were included, highly significant differences occurred in the average weights (Pg. 6, 7) on days 155, 223 and 291, and the average daily weight gains (Pg. 8) were significantly different on day 257. Groups I and III had significantly higher average weights and average daily weight gains than Group C.

Excluding mortalities, Groups I and II yielded US \$548 and US \$144 higher net returns (Pg. 11) than Group C based on 20 calves. Including mortalities, Groups I and II yielded US \$935 and US \$311 higher net returns (Pg. 8) than Group C based on 20 calves.

From these data, it is evident that the use of endoparasitic and ectoparasitic control measures are profitable and feasible, but greater profits

can be obtained when combined with hemoparasitic control methods.

On the basis of data obtained and analyzed from this research project, it can be concluded that control of blood parasites is not only feasible, but economical and productive yielding highly significant net monetary returns. Assuming that the total Colombian cattle population is approximately 20 million, anaplasmosis and babesiosis could directly cause a loss of 25 million dollars or 56 million pounds of beef per year to the Colombian cattle industry based on the experimental information obtained from this project, if these are representative.

## IMMUNE RESPONSE OF CATTLE INOCULATED WITH IRRADIATED

## BABESIA BIGEMINA

## INTRODUCTION

Parasitic protozoan induced immunities demonstrate many features in common with other microbiological infections, but because of the complicated life-cycles of protozoa, the phenomenon is not a simple reaction of the host to a single stage of the parasite. Instead, a variety of responses is evoked by forms which develop in the invertebrate host, by the final products circulating in the blood, and in some instances, by the continually changing antigenic pattern. Apart from the serum antibody response, recent work has indicated that cell-mediated immunity may be important in the host's defenses against certain protozoan parasites (Target, 1968). The inconclusive results obtained with immunization using killed protozoa or protozoan fractions have been attributed to the complex and highly variable antigenic structures of the organisms and the loss of denaturation of the antigens during the preparation of the vaccines. Furthermore, the parasites may have been too rapidly destroyed in the host to elicit a discernible protective effect.

Although immunization against protozoan diseases has been studied in great detail, there has been little progress in the development of reliable vaccines. In fact, immunization has been

put to practical use only against bovine babesiosis, bovine theileriasis, avian coccidiosis, and cutaneous leishmaniasis of man. In these instances, inoculation or exposure of living parasitic protozoa produces infections that result in protection of the host which unfortunately ensures continued transmission of the disease. The narrow margin between the induction of fatal disease and mild infections, resulting in protection, has led to the search for avirulent organisms that retain their immunogenic properties

Irradiation of protozoa interferes with their physiologic processes and frequently inhibits their normal development and multiplication (Giese, 1967). Studies on the effects of ionizing radiations on parasitic protozoa have repeatedly shown that while an extremely high radiation dose is necessary to cause immediate death of the parasite, much lower doses can interfere with infectivity (Kimball, 1955). The immunogenicity of irradiated Babesia rodhaini (Phillips, 1970 & 1971b), Plasmodium spp. (Corradetti, et al., -1968; Nussenzweig, et al., 1969; Sadun, et al., 1969) and Trypanosoma spp. (Duxbury & Sadun, 1969 & 1970; Sanders & Wallace, 1966) in experimental animals (mice, rats, and owl monkeys) has been investigated. These studies have shown that parasites irradiated with a dose which abolished their reproductive potential and ability to produce patent infections may retain their capacity to produce an immune response in a susceptible experimental animal. Therefore, the use of irradiated parasitic protozoa as vaccines

might provide the special immunological properties of living protozoa while suppressing the pathogenic effects.

More information is needed from studies which evaluate the potential use of irradiated parasitic protozoa as vaccines against naturally occurring blood protozoan diseases where interactions among the host, parasite, vector, and environment are involved in promoting a state of stable immunity. Bovine babesiosis which occurs in the warmer areas of all continents offers a unique system to evaluate the use of irradiated vaccines against naturally occurring blood protozoan diseases. Bovine babesiosis is a naturally occurring tick-borne disease caused by species of Babesia which penetrate and destroy erythrocytes. The expert panel at the first Research Coordination Meeting of the joint Food and Agriculture Organization and International Atomic Energy Agency program on the use of isotopes and radiation in parasitology has recommended that radiation studies with Babesia be encouraged in view of their potential value in the development of vaccines (Anon., 1968).

The following series of investigations were undertaken to study the effect of various radiation dosages on the infectivity and immunogenicity of erythrocytic stages of Babesia bigemina. The effect of freezing on the immunogenicity of irradiated B. bigemina was also studied. The studies were conducted toward the ultimate goal of producing irradiated vaccines against bovine babesiosis. . .

## MATERIALS AND METHODS

### Experimental Animals

Three-day-old male Holstein calves were obtained from near Facatativa, Colombia and were hand-raised to 3 months of age on the Tibaitata Instituto Colombiano Agropecuario (ICA) experiment station, in an area free of Boophilus microplus. The calves were housed for the duration of the experiment in a tick-free environment at the ICA Laboratorio de Investigaciones Medicas Veterinarias (LIMV) in Bogota, Colombia. Insect control was maintained by the periodic application of a residual Dichlorvos\* oil base spray in the housing area.

The calves were given a grain ration (see Appendix Table A1) at the rate of 2.7% of their body weight which met the daily nutrient requirements for growth and maintenance of dairy calves suggested by the National Research Council (Anon., 1966). The calves were also given oat silage, salt, and a 1:1 (v/v) mixture of salt and bone meal free choice throughout the experiment.

The calves were screened for the incidence of helminthism and coccidiosis by fecal examination and treated with thiabendazole\*\* and sulfabromomethazine\*\*\* before the experiment began.

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\*Vapona, Shell Colombia, S. A., Bogota.

\*\*Thiabendazole, Merck Sharp & Dohme Quimica de Colombia, S. A., Bogota and Cali, Colombia.

\*\*\*Suldur, R. E. Squibb & Sons, Inter-American Corporation, Cali, Colombia.

The calves were vaccinated against foot-and-mouth disease, virus types O and A, Clostridium chauvoei, Clostridium septicum, Pasteurella multocida, and Pasteurella hemolytica before the experiment began.

Splenectomized calves were used for the isolation and separation of B. bigemina, preparation of irradiated and challenge inocula, subinoculation for the detection of subpatent parasitemia and production of antigens for the CF test. Splenectomy was carried out under general anesthesia using 1 ml per kg of body weight of an aqueous solution of chloral hydrate (42.50 g/l), pentobarbital (9.72 g/l), and magnesium sulfate (11.26 g/l).\*

The spleen was exposed through a left lateral incision in the paralumbar fossa and after ligating the splenic artery and vein, the spleen was removed. Blood smears and CF reactions for Babesia and Anaplasma were determined weekly for at least 5 weeks after splenectomy and prior to inoculation, to insure that they did not carry a latent infection with Babesia or Anaplasma.

#### Experimental Organisms

The B. bigemina used in the following studies was obtained from a splenectomized calf which had an acute tick-transmitted infection of B. bigemina. A 4-month-old splenectomized calf was

\*Equi-Thesis, Jensen-Salsbery Laboratories, Kansas City. No. 64142.

transported by airplane from the ICA-LETV laboratories in Bogota to the northern coastal area of Colombia and placed on a tick-infested pasture at the Turipana ICA experiment station near Monteria.

Daily thin films were made with blood obtained from the jugular vein using 1.3 g/l of disodium ethylenediamine tetraacetate (EDTA) as an anticoagulant. The films were fixed in absolute methanol and stained with a 1 to 20 solution of Giemsa\* for 20 minutes, using phosphate buffered water (pH 7.0-7.1) containing 0.01% alkyl phenoxy polyethoxy ethanol\*\* (APPE) to prepare the stain solution. Stock solutions of M/15  $\text{Na}_2\text{HPO}_4$ , M/15  $\text{NaH}_2\text{PO}_4$ , and 10% APPE were prepared and stored in separate glass stoppered bottles. Fresh buffered water containing 0.01% APPE was prepared weekly by using 39 ml of M/15  $\text{NaH}_2\text{PO}_4$ , 61 ml of M/15  $\text{Na}_2\text{HPO}_4$ , 1 ml of 10% APPE, and 899 ml of distilled water. An electric pH meter\*\*\* was used to determine the pH of the freshly prepared buffered water containing APPE. The films were examined microscopically, using a microscope with an oil immersion objective, for 30 minutes in

\*Giemsa Stain. Gradwohl Laboratories, 3514 Lucas Avenue, St. Louis, Mo. 63155.

\*\*Triton X-100. Rohm and Hass, Independence Mall West, Philadelphia, Pa. 19105.

\*\*\*Beckman Expandometric Model 76. Beckman Instruments, Inc., Scientific Instruments Division, Fullerton, California 92634.

cases when the first appearance of Babesia was thought to be imminent. The films were examined about 3 to 6 mm from the end of the film and transversed from one side of the film to the other to give a constant and representative sample. After B. bigemina was found in blood smears, 180 ml of blood were withdrawn from the jugular vein of the splenectomized calf, using 1.3 g/l of dipotassium EDTA as an anticoagulant. The blood was transported by airplane to the ICA-LIMV laboratories in Bogota for the separation of B. bigemina from B. argentina, B. major, and Anaplasma marginale.

The method of separation of B. bigemina from B. argentina, B. major, and A. marginale involved rapid passage through 5 splenectomized calves and was based on that used by Sergent et al. (1927) and Callow and Hoyte (1961a). The first calf was inoculated with blood carrying several different organisms, and subsequent subinoculations were done soon after blood smears from each calf were found to be positive for B. bigemina. Blood for subinoculation was collected from the jugular vein without using an anticoagulant and each passage was carried out immediately by injecting the blood into the jugular vein of the next splenectomized calf.

The drugs used to treat the Babesia infections were trypan blue and 4,4' -diamidino-diazoaminobenzene diacetate.\* Trypan

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\*Ganaseg. Squibb, Cali, Colombia.

blue was used for suppressing the B. bigemina infection without interfering with the potential B. argentina infection.

A frozen stabilate of B. bigemina was made using a modification of the method reported by Pipano and Senft (1966). Blood containing 2.5% B. bigemina parasitized erythrocytes was collected from the fifth splenectomized calf at the acute stage of the disease by venipuncture using 1.3 g/l disodium EDTA as an anticoagulant. Glycerol was used as a cryoprotective agent, at a final concentration of 11.6%. Fifty ml of the blood-glycerol mixture were dispensed in 60 ml plastic narrow mouth bottles with screw caps\*, and incubated for 30 minutes at 4°C. After incubation, the blood was stored in a dry ice cabinet at -79°C.

#### Preparation of Irradiated and Challenge Inocula

Blood containing B. bigemina was collected from splenectomized calves during the acute stage of babesiosis after inoculation with 50 ml of the frozen stabilate of B. bigemina. Although calves were inoculated or challenged at different times, each inoculum was prepared from splenectomized calves previously inoculated with a standard dose of the same B. bigemina infected blood. Blood containing B. bigemina was collected from the donor splenectomized calves during the acute stage of the disease by venipuncture using 1.3 g/l of dipotassium EDTA as an anticoagulant.

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\*Kimble, Owens-Illinois, Toledo, Ohio 43601.

The number of B. bigemina parasitized erythrocytes per ml of blood was determined from an estimate of the percentage of erythrocytes parasitized and the erythrocyte count.

For irradiation treatment, 120 ml of inoculum were divided equally between 2 plastic Petri dishes, 1.3 cm deep and 9.1 cm in diameter. The depth of inoculum in each Petri dish was approximately 1.0 cm. The inoculum in one Petri dish was exposed to the desired amount of radiation using a cobalt-60 teletherapy unit\*. This 3129-c source delivered a dose rate of approximately 200 Rads/min at an exposure distance of 50 cm. The dose rate was determined by ferrous chemical dosimetry and periodic verification of the dose rate was carried out by means of a dosimeter\*\*. The inoculum in the other Petri dish was placed in an adjacent room for the duration of the irradiation and used as a nonirradiated control.

#### Experimental Procedures

Five experiments were performed to study the effects of various gamma radiation dosages on the infectivity and immunogenicity of erythrocytic stages of B. bigemina in cattle. In experiment

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\*Eldorado 8. Atomic Energy of Canada Limited: Commercial Products, P. O. Box 93, Ottawa, Canada.

\*\*Victoreen r-Meter Mod. No. 570. Victoreen Instrument Division, 10101 Woodland Ave., Cleveland, Ohio 44104.

I, 8 three-month-old calves were divided into 4 groups of 2 calves each. Three groups of calves were given one intravenous inoculation of blood containing  $1 \times 10^9$  B. bigemina parasitized erythrocytes previously exposed to radiation doses of 22, 26, and 30 kRad. One calf in the control group was given one intravenous inoculation of nonirradiated blood containing  $1 \times 10^9$  B. bigemina parasitized erythrocytes and the other control calf received one intravenous inoculation of a similar volume of nonparasitized blood previously exposed to a radiation dose of 30 kRad. All calves in experiment I were challenged intravenously with blood containing  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes 7 weeks after the last inoculation.

In experiment II, 6 three-month-old calves were divided into 3 groups of 2 calves each. Two groups of calves were given one intravenous inoculation of blood containing  $1 \times 10^9$  B. bigemina parasitized erythrocytes previously exposed to radiation doses of 36 and 42 kRad. The groups of calves that failed to show parasitic multiplication and reinvasion of erythrocytes were given a second inoculation in a similar manner 14 days after the first inoculation to enhance any immunizing effect that the irradiated parasites might have had. One calf in the control group was given one intravenous inoculation of nonirradiated blood containing  $1 \times 10^9$  B. bigemina parasitized erythrocytes and the other control calf received 2 intravenous inoculations at a 14 day interval of a similar volume of nonparasitized blood

previously exposed to a radiation dose of 42 kRad.

In experiment III, 4 three-month-old calves were divided into 2 groups of 2 calves each. The 2 groups of calves were given one intravenous inoculation of blood containing  $1 \times 10^9$  B. bigemina parasitized erythrocytes previously exposed to radiation doses of 36 and 42 kRad. The inoculation schedules in experiments II and III were arranged so that all calves received their final inoculation on the same day. All calves in experiments II and III were challenged intravenously with blood containing  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes 3 weeks after the last inoculation.

In experiment IV, 31 four-month-old calves were divided into 4 experimental groups of 4 calves each and 5 control groups of 3 calves each. The 4 experimental groups of calves were given one intravenous inoculation of blood containing  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes previously exposed to radiation doses of 24, 36, 48, and 60 kRad.

The first control group of calves were given one intravenous inoculation of nonirradiated blood containing  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes. To evaluate the possibility that some reduction in parasitemia might be the result of a reduction in the number of viable parasites injected rather than an attenuation of the parasites injected, the second and third control groups of calves received on intravenous inoculation of nonirradiated blood containing  $1 \times 10^7$  and  $1 \times 10^4$  B. bigemina parasitized

erythrocytes, respectively. To test the hypothesis that living erythrocytic stages of B. bigemina exposed to a radiation dose sufficient enough to prevent progressive parasitemias are more immunogenic than non-living parasites, the fourth control group of calves were given one intravenous inoculation of blood containing  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes previously heat inactivated in a water bath at  $56^{\circ}\text{C}$  for 30 minutes. The fifth control group of calves received one intravenous inoculation of a similar volume of nonparasitized blood previously exposed to a radiation dose of 36 kRad. All calves in experiment IV were challenged intravenously with blood containing  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes 4 weeks after their last inoculation.

Experiment V was undertaken to study the effect of freezing on the immunogenicity of irradiated erythrocytic stages of B. bigemina. An aliquot of blood containing B. bigemina parasitized erythrocytes previously exposed to a radiation dose of 60 kRad for the fourth experimental group in experiment IV was frozen using the method previously described for the preservation of B. bigemina. Four 4-month-old calves were given one intravenous inoculation of irradiated blood containing  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes previously frozen for 48 hours. The inoculated calves in experiment V were challenged intravenously with blood containing  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes 26 days after the last inoculation with an aliquot of the same challenge inoculum used in experiment IV and on the same day as in experiment IV.

### Assessment of Reactions

#### Parasitemia

Combination thin and thick blood films. The calves were examined daily for the presence of parasitized erythrocytes by the use of combination thin and thick blood films. The blood films were made with blood obtained from the jugular vein using 1.3 g/l of disodium EDTA as an anticoagulant. The thin blood films was extended over 1/2 the length of the microscope slide and rapidly dried. The method used for the preparation of the thick film on the opposite end of the microscope slide was based on a technique described by Mahoney and Saal (1961).

The thin blood film was fixed with absolute methyl alcohol. The slide was held in a slanted position with the thin film down and a few drops of absolute methyl alcohol placed on the thin film. To prevent alcohol or alcohol fumes from contacting the thick film, the slide was placed in a vertical position with the thick film up until the methyl alcohol had evaporated. The slides were then placed in a staining rack and kept at this stage for further processing.

Just before staining the slides were placed in an incubator for

drying was required to ensure that the thick films did not wash off during the staining process. To prevent deterioration of the thick films, the combination thin and thick films were stained soon after final drying.

The method used for the staining of the combination thin and thick films was based on a staining technique described for Plasmodium spp. in man (Anon., 1970). The thin and thick films were placed in a 1 to 50 solution of Giemsa stain for 45 minutes to lyse the unfixed erythrocytes in the thick film and to stain the blood films. Phosphate buffered water (pH 7.0-7.1) containing 0.01% APPE was used to prepare the stain solution.

Immediately following staining, the thin and thick films were rinsed briefly by dipping the slides in a staining dish filled with buffered water (pH 7.0-7.1) containing 0.01% APPE. A rubber band was placed around the staining rack to hold the slides in place. The staining rack was placed in a vertical position with only the thick film immersed in the same buffered water containing APPE for an additional 3 to 5 minutes. The slides were dried in the vertical position with the thick film down.

For confirmation of Babesia parasitemia on thick films, it was necessary to resolve the objects as having a red nucleus and blue cytoplasm and to find some parasites that were typically pyriform and arranged in pairs. An estimate of the number of parasites per  $\text{mm}^3$  of blood on thick films was made from the number

of parasites seen on 20 fields, and from an estimate of the number of leukocytes present on 20 fields, and from an estimate of the number of leukocytes per  $\text{mm}^3$  of blood. The number of parasites per  $\text{mm}^3$  of blood was then converted to percent parasitized erythrocytes from an estimate of the number of erythrocytes per  $\text{mm}^3$  of blood.

Subinoculations. Subinoculations were performed the day of challenge from 4 groups in experiment IV which were inoculated with parasitized erythrocytes, but failed to demonstrate detectable parasitemias to determine whether subpatent parasitemia had resulted. Subinoculations from the 4 groups were accomplished by collecting 100 ml of blood from each calf in each group by venipuncture using 1.3 g/l disodium EDTA as an anticoagulant and injecting the blood intravenously by group into 4 splenectomized calves.

#### Packed Cell Volume

Daily packed cell volumes were determined by the microhematocrit method with blood obtained from the jugular vein using 1.3 g/l of disodium EDTA as an anticoagulant. Plain capillary tubes 75 mm x 1.2 mm were filled to approximately 1 cm from the end and the vacant end of the tube was sealed by holding it in a flame of a high temperature gas burner. The filled capillary tubes were

then centrifuged\* for 5 minutes at 11,500 rpm and the packed cell volumes read directly from a graphic reader\*\*.

#### Rectal Temperatures and Body Weights

Daily rectal temperatures were measured each morning throughout the experiment. All calves were weighed\*\*\* weekly following a 12 hour withdrawal from feed.

#### Babesial Complement Fixing Antibody Levels

General. Determination of babesial CF serum antibody levels was performed by a microtiter procedure similar to that described by Hidalgo and Dimopoulos (1967). Serum samples were collected from all calves twice a week for the CF microtiter procedure. Whole blood was collected by jugular puncture, allowed to clot, and serum recovered after centrifugation\*\*\*\* at 2,500 rpm for 20 minutes.

The CF microtiter procedure was conducted in polished transparent plastic trays containing 12 rows of 8 wells each. Reagents

\*International Micro-Capillary Centrifuge, Model MB. International Equipment Company, Needham Hts., Mass. 02194.

\*\*International Micro-Capillary Reader. International Equipment Company, Needham Hts., Mass. 02194.

\*\*\*Ranger Balance, Model SF1. Ranger Mating Mfg., Washington C.H., Ohio 43160

\*\*\*\*International Centrifuge, Size 2, Model K. International Equipment Co., Needham Hts., Mass. 02194.

except serums were added to wells with dropping pipettes\* which delivered 0.025 ml. per drop. Serums were transferred to the wells in the test tray and serially diluted with the microloops\*\* which delivered 0.025 ml. Incubation of trays was conducted at 37°C after being covered with an airtight plate sealer to prevent excessive evaporation of reagents.

Veronal buffer was used to dilute all reagents in the CF microtiter procedure. It was prepared and stored as described in the standard anaplasmosis CF procedure (Anon., 1958). Ovine erythrocytes for the hemolytic system were collected, stored, and washed as previously described (Anon., 1958). For conducting the CF microtiter procedure, 2.0% suspensions of ovine erythrocytes were prepared as described in the standard anaplasmosis CF procedure (Anon., 1958).

Hemolysin titration. The following reagents were prepared to titrate hemolysin: (1) 2% ovine erythrocytic suspension, (2) 1:25 dilution of a commercially produced complement\*\*\*, and (3) 1:1,000 dilution of a commercially produced hemolysin\*\*\*\*. The

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\*Microtiter Pipette Dropper. Cooke Engineering Company, Medical Research Division, 900 Slaters Lane, Alexandria, Va. 22314.

\*\*Microtiter Loops. Cooke Engineering Company, Medical Research Division, 900 Slaters Lane, Alexandria, Va. 22314.

\*\*\*TBL Lyophilized Guinea Pig Complement. Texas Biological Lab. Inc., P.O. Box 722, Fort Worth, Texas 76101.

\*\*\*\*Sycco Sheep Cell Hemolysin. The Sylvania Co., Millburn, New Jersey 07041.

1:1,000 dilution of hemolysin was further diluted and titrated according to the scheme previously described (Anon., 1958). For the preparation of the hemolytic system, equal amounts of the 2% suspension of ovine erythrocytes and diluted titrated hemolysin were mixed and stored at 4-6°C for 1 day before using. A unit of hemolysin was defined as the highest dilution producing complete hemolysis of an equal volume of a 2% ovine erythrocyte suspension within 45 minutes at 37°C in the presence of an excess of complement.

Complement titration. The following reagents were prepared to titrate the complement: (1) 2% ovine erythrocytic suspension, (2) hemolysin dilution (2 units/ml), and (3) 1:25 dilution of commercially produced complement. The complement was further diluted and titrated according to the scheme previously described (Anon., 1958). A unit of complement was defined as the highest dilution producing complete hemolysis of a equal volume of 2% ovine erythrocytic suspension in the presence of an equal volume of hemolysin (2 units/ml) within 45 minutes at 37°C.

Preparation of antigen. Blood containing 23% B. bigemina parasitized erythrocytes was collected by carotid artery canulation from a splenectomized calf during the acute stage of babesiosis after inoculation with 50 ml of the frozen stabilate of B. bigemina. Blood was collected in sterile 2 l Erlenmeyer flasks using 1.3 g/l disodium EDTA as an anticoagulant. After collection, the blood was placed in a refrigerator for 3 hours at 4-6°C. The plasma was

removed after centrifugation\* at 2,500 rpm (1020 xg) for 20 minutes at 2-4°C, and the erythrocytes were washed twice with sterile physiological saline at 4-6°C.

Babesia bigemina antigens for the CF microtiter procedure were prepared from the washed erythrocytes by a method described by Mahoney (1967c). The washed erythrocytes were lysed with 10 volumes of 0.35% sodium chloride and the mixture was centrifuged at 5,600 rpm (5,000 xg) for 30 minutes at 2-4°C. The sediment was washed and resuspended in an equal volume of the same diluent and examined microscopically in Giemsa stained smears. Distilled water extracts were prepared by mixing one volume of parasite suspension with 2 volumes of distilled water, shaking vigorously for 2 minutes, and then centrifuging at 5,600 rpm (5,000 xg) for 30 minutes at 4°C. The supernatant fluids were numbered serially in order of collection, placed in ampoules in 3 ml portions and stored in a dry ice cabinet at -79°C. The third extract was strongly antigenic and was the B. bigemina antigen used in the CF microtiter procedure.

Antigen titration. The following reagents were prepared to titrate the antigen: (1) 2% ovine erythrocytic suspension, (2) inactivated positive serums (3) inactivated negative serums, (4)

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\*Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge. Ivan Sorvall Inc., Newtown, Conn. 06470.

hemolysin (2 units/ml), (5) complement (2 units/ml), and (6) antigen dilution of an approximate strength that would cause complete CF within the range of the titration procedure. Antigens were titrated against a group of 6 positive and 2 negative reference serums according to the scheme previously described (Anon., 1958). The positive serums were from animals that had recovered from blood-induced infections. The serums were taken from 1 to 4 months after infection and were inactivated in a water bath at 56°C for 30 minutes. A unit of antigen was defined as the highest dilution causing complete fixation of an equal volume of complement (2 units/ml) in the presence of the weakest positive reference serum diluted 1:5.

Complement fixation microtiter procedure. A 2% ovine erythrocytic suspension and dilutions of antigen, complement, and hemolysin each containing 2 units/ml were prepared to conduct the microtiter procedure. In addition, a 1:2.5 dilution of each serum sample was prepared and inactivated in a water bath at 56°C for 30 minutes. Titrations in the trays were prepared by first adding 0.025 ml of buffer to each of 8 wells of 1 row for each sample. A 0.025 ml loopful of the 1:2.5 dilution of serum was added to the first well and serially diluted through the eighth well. A 0.025 ml drop of antigen and 0.025 ml drop of complement were then added to wells 1 through 8. The serums being tested were then allowed to react with the antigen and complement for 1 hour in a

water bath at 37°C. Subsequently, 0.05 ml of the hemolytic system consisting of an equal volume of diluted hemolysin and 2% ovine erythrocytic suspension was added and the trays were incubated for 1 more hour. Serum controls on all serums tested were prepared and read in tubes according to the scheme previously described (Anon., 1953).

Positive and negative serum standards and antigen, complement, and hemolytic system controls also were prepared. The antigen control consisted of 0.025 ml of complement, 0.025 ml of antigen, 0.025 ml of buffer, and 0.05 ml of hemolytic system. The complement control consisted of 0.025 ml of complement, 0.05 ml of buffer, and 0.05 ml of hemolytic system. The hemolytic system control consisted of 0.075 ml of buffer and 0.05 ml of hemolytic system. After final incubation, trays were stored 2 hours at 4°C to allow unhemolyzed cells to form sediments in the bottom of the wells. Reactions were read by placing the transparent trays on a concave mirror stand. The titer of the serum was considered to be the highest dilution having complete fixation

## RESULTS

Separation of *B. bigemina* from *B. argentina*, *B. major*,  
and *A. marginale*

The splenectomized calf placed on a tick-infested pasture at the Turipana ICA station was found to be heavily infested with *B. microplus* ticks in the larval stage within 3 days, ticks in the nymphal stage within 8 days, and ticks in the adult stage within 14 days. After 16 days, the body temperature of the calf rose to 40.4°C and *B. argentina* was found in thin blood smears stained with Giemsa. Two days later, *B. bigemina* was found in blood smears. The results of the rapid passages of *B. bigemina* through 5 splenectomized calves to separate *B. bigemina* from contaminating organisms are summarized in Table I.

*Babesia bigemina* was found in blood smears from the first calf after 36 hours. A *B. argentina* infection, which developed in the first calf 4 days after the inoculation with blood, was treated with 4,4'-diamidino-diazoaminobenzene diacetate, 3 mg/kg for 5 days. *Anaplasma marginale* was present in smears from the first calf 44 days after inoculation.

*Babesia bigemina* was found in blood smears from the second calf after 30 hours. A *B. argentina* infection, which developed in the second calf 4 days after the inoculation with blood, was treated with 4,4'-diamidino-diazoaminobenzene diacetate, 3 mg/kg for the following 2 days. The second calf died from babesiosis 1

day after the last treatment.

The third calf was found to be positive for B. bigemina 24 hours after inoculation with 12 ml of blood from the second calf. The third calf was subsequently treated with an intravenous injection of 20 ml of 1% trypan blue, which suppressed the B. bigemina infection. Babesia argentina and B. major infections, which developed in the third calf 15 days after inoculation with blood, were treated the following day with 4,4'-diamidino-diazoaminobenzene diacetate, 3 mg/kg. The third calf died from babesiosis 1 day after treatment.

The fourth calf was found to be positive for B. bigemina 37½ hours after inoculation with 12 ml of blood from the third calf. The procedure of treating the fourth calf with trypan blue was repeated.

Babesia bigemina was found in blood smears from the fifth calf after 57½ hours and within an hour, 12 ml of blood were taken for intravenous passage into an intact calf. A frozen stabilate of B. bigemina was made from the fifth calf 4 days after inoculation when the parasitemia had reached 2.5%. The day following freezing, 50 ml of the frozen stabilate was inoculated intravenously into a second intact calf.

The first intact calf was found to be positive for B. bigemina 2 days after inoculation. The second intact calf was found to be positive for B. bigemina 5 days after inoculation. The intact calves did not require treatment with trypan blue. Babesia

argentina, B. major, and A. marginale were not found in any smear from either of the 2 intact calves nor in any smear from the fourth and fifth splenectomized calves for 2 months after inoculation. Babesia argentina and B. major were therefore eliminated as contaminating organisms after 4 passages. The second and third splenectomized calves died of babesiosis which made it impossible to estimate at which point A. marginale failed to be passaged. Photographs of B. bigemina on thin and thick blood films are shown in Figures 1 and 2, respectively. Babesia bigemina used for the preparation of antigens for the CF microtiter procedure is shown in Figure 3.

#### Experiment I

The first experiment was undertaken to determine the effect of gamma radiation dosages of 22, 26, and 30 kRad on the infectivity and immunogenicity of B. bigemina in calves.

The results summarized in Figures 4 to 8 and Table 2 show that calves infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to dosages up to and including 30 kRad developed progressive parasitemias which were delayed in comparison to the calves inoculated with nonirradiated B. bigemina parasitized erythrocytes. The average prepatent periods were  $7\frac{1}{2}$ , 9, and 10 days for calves infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 22, 26, and 30 kRad, respectively. The average prepatent periods for calves infected with nonirradiated

B. bigemina parasitized erythrocytes were 2 days. The average maximum parasitemias were 0.29, 0.13, 0.04, and 0.08% for calves infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad, respectively. The average maximum morning rectal temperatures were 40.6, 39.5, 41.0, and 40.0°C for calves infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad, respectively. The average minimum packed cell volumes were 26, 28, 30, and 32% for calves infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad, respectively. The average maximum CF titers were 1:320, 1:227, 1:113, and 1:113 during 28 days following inoculation with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad, respectively. The average daily gains were 423, 840, 572, and 786 g/day for 28 days following inoculation with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad, respectively. The average prepatent periods, parasitemias, maximum temperatures, minimum packed cell volumes, average daily gains, and immune responses were similar in calves infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad following challenge with  $1 \times 10^{10}$  nonirradiated B. bigemina.

One calf inoculated with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 30 kRad died 34 days after inoculation with chronic bacterial pneumonia. One calf, 7 weeks after being inoculated with blood containing  $1 \times 10^9$  B. bigemina parasitized

erythrocytes exposed to 26 kRad, developed a severe systemic reaction following inoculation of blood containing  $1 \times 10^{10}$  non-irradiated B. bigemina parasitized erythrocytes. Within 2 minutes of completion of the injection, the calf suffered intense respiratory distress, collapsed on its side and showed nystagmus. Coughing produced a cream colored frothy fluid which was mixed with blood. The calf breathed more easily after 5 minutes and stood up after 15 minutes. Its posture was characteristic of dyspneic animals with the neck extended, head lowered, mouth opened, and tongue protruded. Abdominal respiratory movements were marked and grunting accompanied expirations.

#### Experiments II and III

The second experiment was undertaken to determine the effect of gamma radiation dosages of 36 and 42 kRad on the infectivity and immunogenicity of B. bigemina in calves given 2 inoculations at 14 day intervals. The results of experiment II summarized in Figures 9-12 and Table 2 (p. 71) show that calves did not develop progressive parasitemias following the first inoculation of  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to dosages of 36 and 42 kRad. The same calves, however, developed progressive parasitemias following the second inoculation of  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to dosages of 36 and 42 kRad. The average prepatent periods were 8 and 9 days for calves infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes

exposed to 36 and 42 kRad, respectively. The average prepatent periods for calves infected with nonirradiated B. bigemina parasitized erythrocytes were 2 days.

The third experiment was undertaken to determine the effect of gamma radiation dosages of 36 and 42 kRad on the infectivity and immunogenicity of B. bigemina in calves given one inoculation.

The results of experiment III summarized in Figures 13 and 14 and Table 2 (p. 71) show that calves infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to dosages of 36 and 42 kRad developed progressive parasitemias which were delayed in comparison to calves inoculated with nonirradiated B. bigemina. The prepatent period was 7 days for all calves infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 36 and 42 kRad. The average prepatent period for calves infected with nonirradiated B. bigemina parasitized erythrocytes was 2 days.

The average maximum parasitemias were 0.29, 0.02, and 0.01% for calves in experiments II and III infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad, respectively. The average maximum morning rectal temperatures were 40.6, 39.8, and 39.7°C for calves in experiments II and III inoculated with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad, respectively. The average minimum packed cell volumes were 26, 25, and 29% for calves in experiments II and III inoculated with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad, respectively. The average

maximum CF titers were 1:320, 1:33, and 1:37 following inoculation with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad, respectively. The average daily gains were 423, 742, and 576 g/day for 28 days following inoculation with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad, respectively. The average prepatent periods, parasitemias, maximum temperatures, minimum packed cell volumes, average daily gains and immune responses were similar in calves infected with  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad following challenge with  $1 \times 10^{10}$  nonirradiated B. bigemina.

Two calves in experiment II, 21 days after being inoculated the second time with blood containing  $1 \times 10^9$  B. bigemina parasitized erythrocytes exposed to 36 kRad developed severe systemic reactions. Within 2 minutes of completion of the injections, the calves suffered intense respiratory distress. One calf collapsed on its side and showed nystagmus. Coughing produced a cream colored frothy fluid which was mixed with blood and respiration ceased 10 minutes after injection. Post-mortem examination of the calf revealed lesions which were confined largely to the respiratory system. There were severe intra-alveolar and interstitial edema and emphysema with intra-alveolar hemorrhage. The trachea and major bronchi contained a cream colored frothy fluid which was bloodstained. The second calf breathed more easily after 5 minutes and stood up after 15 minutes. Its posture was

characteristic of dyspneic animals with the neck extended, head lowered, mouth opened, and tongue protruded. Abdominal respiratory movements were marked and grunting accompanied expirations.

#### Experiments IV and V

The fourth experiment was undertaken to determine the effect of gamma radiation dosages of 24, 36, 48, and 60 kRad on the infectivity and immunogenicity of B. bigemina in calves.

The results of experiment IV summarized in Figures 15-23 and Tables 2 (p. 71) and 3 show that calves inoculated with B. bigemina parasitized blood exposed to 24 kRad developed progressive parasitemias which were delayed in comparison to calves inoculated with  $1 \times 10^{10}$  nonirradiated B. bigemina. Three out of 4 calves receiving parasitized blood irradiated at 36 kRad did not develop progressive parasitemias. Progressive infections were prevented by exposure to irradiation at 48 and 60 kRad. The average prepatent periods were 6.5 and 13 days for calves infected with  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes exposed to 24 and 36 kRad, respectively. The average prepatent period for calves infected with  $1 \times 10^{10}$  nonirradiated B. bigemina parasitized erythrocytes was 2 days. The average prepatent period for calves infected with  $1 \times 10^7$  nonirradiated B. bigemina parasitized erythrocytes was 5.7 days. Calves inoculated with  $1 \times 10^4$  nonirradiated B. bigemina parasitized erythrocytes did not develop progressive parasitemias.

The average maximum parasitemias were 3.2 and 0.03% for calves infected with  $1 \times 10^{10}$  and  $1 \times 10^7$  nonirradiated B. bigemina parasitized erythrocytes, respectively. The average maximum parasitemias were 0.11 and 0.002% for calves infected with  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes exposed to 24 and 36 KRad, respectively.

The average maximum morning rectal temperatures were 41.5 and 40.5°C. for calves infected with  $1 \times 10^{10}$  and  $1 \times 10^7$  nonirradiated B. bigemina parasitized erythrocytes, respectively. The average maximum morning rectal temperatures were 40.3 and 40.1°C for calves infected with  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes exposed to 24 and 36 KRad, respectively.

The average minimum packed cell volumes were 18 and 27% for calves infected with  $1 \times 10^{10}$  and  $1 \times 10^7$  nonirradiated B. bigemina parasitized erythrocytes, respectively. The average minimum packed cell volumes were 26 and 30% for calves infected with  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes exposed to 24 and 36 KRad, respectively.

The average maximum CF titers were 1:320 and 1:201 for calves infected with  $1 \times 10^{10}$  and  $1 \times 10^7$  nonirradiated B. bigemina parasitized erythrocytes, respectively. The average maximum CF titers were 1:135 and 1:80 for calves infected with  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes exposed to 24 and 36 KRad, respectively.

The average daily gains were 423 and 798 g for 28 days following inoculation with  $1 \times 10^{10}$  and  $1 \times 10^7$  nonirradiated B. bigemina parasitized erythrocytes, respectively. The average daily gains were 661 and 857 g for calves infected with  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes exposed to 24 and 36 KRad, respectively.

The fifth experiment was undertaken to determine the effect of gamma radiation of 60 KRad and freezing on the infectivity and immunogenicity of B. bigemina in calves. The results of experiment V summarized in Figure 24 and Table 3 (147) show that calves inoculated with B. bigemina parasitized blood exposed to 60 KRad and frozen did not develop progressive parasitemias.

The results of experiments IV and V show that all calves developed progressive parasitemias with similar prepatent periods following challenge with  $1 \times 10^{10}$  nonirradiated B. bigemina parasitized erythrocytes. The average maximum parasitemias were 0.0006, 0.004, 0.5, 0.8, and 0.6% following challenge for calves inoculated 4 weeks previously with  $1 \times 10^{10}$  nonirradiated B. bigemina,  $1 \times 10^7$  nonirradiated B. bigemina,  $1 \times 10^4$  nonirradiated B. bigemina, and 10 ml normal blood irradiated at 36 KRad, respectively. One calf inoculated 4 weeks before challenge with 12 ml normal blood irradiated at 36 KRad died from acute babesiosis following challenge with  $1 \times 10^{10}$  nonirradiated B. bigemina parasitized erythrocytes. The average maximum parasitemias were 0.003, 0.02, 0.1, 0.03, and 0.06% following challenge for calves inoculated 4 weeks

previously with  $1 \times 10^{10}$  B. bigemina irradiated at 24, 36, 48, 60, and 60 kRad (frozen), respectively.

The average maximum morning rectal temperatures were 40.2, 40.2, 41.3, 41.1, and 41.4°C following challenge for calves inoculated 4 weeks previously with  $1 \times 10^{10}$  nonirradiated B. bigemina,  $1 \times 10^7$  nonirradiated B. bigemina,  $1 \times 10^4$  nonirradiated B. bigemina,  $1 \times 10^{10}$  heat inactivated B. bigemina, and 10 ml normal blood irradiated at 36 kRad, respectively. The average maximum morning rectal temperatures were 39.7, 40.0, 39.9, 39.9, and 40.0°C following challenge for calves inoculated 4 weeks previously with  $1 \times 10^{10}$  B. bigemina irradiated at 24, 36, 48, 60, and 60 kRad (frozen), respectively.

The average minimum packed cell volumes were 29, 28, 11, 19, and 10% following challenge for calves inoculated 4 weeks previously with  $1 \times 10^{10}$  nonirradiated B. bigemina,  $1 \times 10^7$  nonirradiated B. bigemina,  $1 \times 10^4$  nonirradiated B. bigemina,  $1 \times 10^{10}$  heat inactivated B. bigemina and 10 ml normal blood irradiated at 36 kRad, respectively. The average minimum packed cell volumes were 27, 26, 24, 25, and 23% following challenge for calves inoculated 4 weeks previously with  $1 \times 10^{10}$  B. bigemina irradiated at 24, 36, 48, 60, and 60 kRad (frozen), respectively.

The average maximum CF titers were 1:201, 1:127, 1:160, 1:640, and 1:227 following challenge for calves inoculated 4 weeks previously with  $1 \times 10^{10}$  nonirradiated B. bigemina,  $1 \times 10^7$  nonirradiated B. bigemina,  $1 \times 10^4$  nonirradiated B. bigemina,  $1 \times 10^{10}$

heat inactivated B. bigemina and 10 ml normal blood irradiated at 36 KRad, respectively. The average maximum CF titers were 1:134, 1:201, 1:380, 1:269, and 1:640 following challenge for calves inoculated 4 weeks previously with  $1 \times 10^{10}$  B. bigemina irradiated at 24, 36, 48, and 60 KRad (frozen), respectively.

The average daily gains were 887, 976, 813, 815, and 982 g for 28 days following challenge for calves inoculated 4 weeks previously with  $1 \times 10^{10}$  nonirradiated B. bigemina,  $1 \times 10^7$  nonirradiated B. bigemina,  $1 \times 10^4$  nonirradiated B. bigemina,  $1 \times 10^{10}$  heat inactivated B. bigemina, and 10 ml normal blood irradiated at 36 KRad, respectively. The average daily gains were 1093, 1060, 947, 938, and 822 g for 28 days following challenge for calves inoculated 4 weeks previously with  $1 \times 10^{10}$  B. bigemina irradiated at 24, 36, 48, 60, and 60 KRad (frozen), respectively.

One calf in experiment V developed a severe systemic reaction following inoculation with previously frozen B. bigemina parasitized blood exposed to 60 KRad. Within 2 minutes of completion of the injection, the calf suffered intense respiratory distress, collapsed to its side, and showed nystagmus. The calf died 36 hours following injection. Post-mortem examination of the calf revealed lesions which were confined largely to the respiratory system. The lungs had severe intra-alveolar and interstitial edema and emphysema with intra-alveolar hemorrhage. The trachea and major bronchi contained a cream colored frothy fluids. Submucosal petechia were present in the trachea. Visceral and

parietal subpleural petechia were numerous. The nasal turbinate mucosa was congested as were the bronchial and mediastinal lymph nodes.

Six calves in experiments IV and V developed severe systemic reactions within 2 minutes following challenge with blood containing  $1 \times 10^{10}$  nonirradiated B. bigemina parasitized erythrocytes. Three of these calves (one inoculated 4 weeks before challenge with  $1 \times 10^{10}$  B. bigemina irradiated at 22 kRad, one inoculated 4 weeks before challenge with  $1 \times 10^{10}$  B. bigemina irradiated at 60 kRad, and one inoculated with 10 ml normal blood irradiated at 36 kRad) suffered intense respiratory distress. These calves breathed more easily after 15 minutes. Their postures were characteristic of dyspneic animals with the neck extended, head lowered, mouth opened, and tongue protruded. Abdominal respiratory movements were marked and grunting accompanied expirations. The other 3 calves (one inoculated 4 weeks before challenge with  $1 \times 10^{10}$  B. bigemina irradiated at 36 kRad, one inoculated 4 weeks before challenge with  $1 \times 10^4$  nonirradiated B. bigemina and one inoculated 4 weeks before challenge with  $1 \times 10^{10}$  B. bigemina previously irradiated at 60 kRad and frozen) suffered intense respiratory distress, collapsed, and showed nystagmus. Coughing produced cream colored frothy fluids mixed with blood. These calves died within 24 hours following injection. Post-mortem examination revealed lesions which were confined largely to the respiratory system, which included severe

intra-alveolar and interstitial edema and emphysema with intra-alveolar hemorrhage of the lung. The trachea and major bronchi contained a hemorrhagic cream colored frothy fluid.

Table 2. Characteristics of *Babesia bigemina* Infections Produced with Infected Blood Subjected to Different Radiation Doses. Summary of Experiments I, II, III and IV.

Exp. No.	Radiation Dose of Inoculum in Kilorads	No. of Parasitized Erythrocytes Inoculated	Reaction After Inoculation of Irradiated <i>B. bigemina</i> Parasitized Erythrocytes <sup>a</sup>							Deaths
			No. Calves with Parasitemia / No. Calves Inoculated <sup>b</sup>	Prepatent Periods in Days	Maximum % Parasitized Erythrocytes	Maximum Temperature in °C	Minimum Packed Cell Volume in %	Maximum Complement Fixation Titer	Average Daily Gain in g/day	
IV	0 (controls)	1 x 10 <sup>10</sup>	3/3	2.0 (2-2) <sup>c</sup>	3.2 (0.4-8.0)	41.5 (41.3-41.6)	18 (13-28)	1:320 (1:160-1:640)	423 (71-821)	0
I + II	0 (controls)	1 x 10 <sup>9</sup>	2/2	2.0 (2-2)	0.29 (0.15-0.42)	40.6 (40.1-41.0)	26 (23-29)	1:320 (1:320-1:320)	423 (256-589)	0
IV	0 (controls)	1 x 10 <sup>7</sup>	3/3	5.7 (5-6)	0.03 (0.008-0.06)	40.5 (40.3-40.7)	27 (24-32)	1:201 (1:160-1:320)	798 (643-911)	0
IV	0 (controls)	1 x 10 <sup>4</sup>	0/3	NONE	NONE	39.7 (39.2-40.0)	27 (24-28)	Neg. (Neg.-Neg.)	863 (643-1026)	0
I	22	1 x 10 <sup>9</sup>	2/2	7.5 (7-8)	0.13 (0.09-0.18)	39.5 (39.5-39.5)	28 (26-29)	1:227 (1:160-1:320)	840 (840-840)	0
IV	24	1 x 10 <sup>10</sup>	4/4	6.5 (6-8)	0.11 (0.008-0.40)	40.3 (39.5-40.7)	26 (22-30)	1:135 (1:80-1:320)	661 (500-786)	0
I	26	1 x 10 <sup>9</sup>	2/2	9.0 (8-10)	0.04 (0.005-0.08)	41.0 (40.9-41.1)	30 (28-32)	1:113 (1:80-1:160)	572 (536-607)	0
I	30	1 x 10 <sup>9</sup>	2/2	10.0 (10-10)	0.08 (0.005-0.15)	40.0 (39.3-40.7)	32 (30-33)	1:113 (1:80-1:160)	786 (786-786)	0
II + III	36	1 x 10 <sup>9</sup>	4/4	7.5 (6-10)	0.02 (0.01-0.04)	39.8 (39.6-40.1)	25 (21-28)	1:33 (Neg.-1:320)	742 (476-857)	0
IV	36	1 x 10 <sup>10</sup>	1/4	13.0 (13-13)	0.002 (0.002-0.002)	40.1 (39.7-40.3)	30 (26-33)	1:13 (Neg.-1:80)	790 (536-1005)	0
II + III	42	1 x 10 <sup>9</sup>	4/4	8.0 (7-10)	0.01 (0.003-0.02)	39.7 (39.5-40.2)	29 (28-31)	1:37 (Neg.-1:320)	676 (238-893)	0
IV	48	1 x 10 <sup>10</sup>	0/4	NONE	NONE	40.1 (39.6-41.0)	31 (29-34)	1:2 (Neg.-1:5)	875 (661-932)	0
IV	60	1 x 10 <sup>10</sup>	0/4	NONE	NONE	40.6 (40.0-40.9)	30 (28-31)	1:3 (Neg.-1:20)	857 (786-1018)	0

<sup>a</sup> Characteristics of reactions based on data collected for 28 days post inoculation.

<sup>b</sup> Detected by either thick blood films or subinoculations.

<sup>c</sup> Range of means.

Table 3. Immune Response of Calves Inoculated Once with Irradiated *Babesia bigemina* and Challenged 4 Weeks Later with Nonirradiated *B. bigemina*. Summary of Experiments IV and V.

Exp. No.	Treatment of Inoculum	Size of Inoculum	Reaction After Challenge with $1 \times 10^{10}$ Unirradiated <i>B. bigemina</i> Parasitized Erythrocytes <sup>a</sup>							
			No. Calves with Parasitemia / No. Calves Challenged	Prepatent Period in Days	Maximum % Parasitized Erythrocytes	Maximum Temperature in °C	Minimum Packed Cell Volume in %	Maximum Complement Fixation Titer	Average Daily Gain in g/day	Deaths
IV	0 kRad (Control)	$1 \times 10^{10}$ <i>B. bigemina</i>	3/3	1.0 (1-1) <sup>b</sup>	0.006 (0.001-0.008)	40.2 (39.8-40.5)	29 (28-31)	1:201 (1:80-1:320)	887 (786-964)	0
IV	0 kRad (Control)	$1 \times 10^7$ <i>B. bigemina</i>	3/3	1.7 (1-3)	0.004 (0.002-0.008)	40.2 (40.1-40.3)	28 (28-30)	1:127 (1:80-1:160)	976 (893-1036)	0
IV	0 kRad (Control)	$1 \times 10^4$ <i>B. bigemina</i>	3/3	1.0 (1-1)	0.5 (0.5-0.5)	41.3 (40.9-41.7)	11 (10-12)	1:160 (1:160-1:160)	813 (696-929)	1 <sup>c</sup>
IV	Heat Inact. (Control)	$1 \times 10^{10}$ <i>B. bigemina</i>	3/3	1.0 (1-1)	0.8 (0.4-1.0)	41.1 (39.8-41.8)	19 (16-22)	1:640 (1:640-1:640)	815 (482-1107)	0
IV	35 kRad (Control)	30 ml Normal Blood	3/3	1.0 (1-1)	0.6 (0.1-1.0)	41.4 (41.0-41.7)	10 (3-14)	1:227 (1:160-1:320)	982 (766-1179)	1 <sup>d</sup>
IV	24 kRad	$1 \times 10^{10}$ <i>B. bigemina</i>	4/4	1.0 (1-1)	0.003 (0.002-0.005)	39.7 (39.6-40.0)	27 (25-30)	1:134 (1:80-1:160)	1093 (1000-1264)	0
IV	36 kRad	$1 \times 10^{10}$ <i>B. bigemina</i>	4/4	2.0 (1-4)	0.02 (0.003-0.04)	40.0 (39.4-40.5)	25 (26-27)	1:201 (1:160-1:320)	1060 (839-1214)	1 <sup>c</sup>
IV	48 kRad	$1 \times 10^{10}$ <i>B. bigemina</i>	4/4	1.0 (1-1)	0.1 (0.01-0.3)	39.9 (39.6-40.4)	24 (21-27)	1:380 (1:320-1:640)	947 (875-1036)	0
IV	60 kRad	$1 \times 10^{10}$ <i>B. bigemina</i>	4/4	1.5 (1-2)	0.03 (0.01-0.06)	39.9 (39.7-40.1)	25 (23-27)	1:269 (1:160-1:640)	938 (750-1143)	0
V	60 kRad (Frozen)	$1 \times 10^{10}$ <i>B. bigemina</i>	2/2	1.0 (1-1)	0.06 (0.05-0.07)	40.0 (40.0-40.0)	23 (21-25)	1:640 (1:640-1:640)	822 (768-875)	1 <sup>c</sup>

<sup>a</sup> Characteristics of reactions based on data collected for 28 days post challenge.

<sup>b</sup> Range of means.

<sup>c</sup> Death due to acute anaphylaxis following challenge.

<sup>d</sup> Death due to acute babesiosis following challenge.

Table 1. The Separation of B. bigemina from B. argentina, B. major, and A. marginale by Rapid Passage through 5 Splenectomized Calves.

Passage Level	Inoculum	Incubation	Parasite	Time of Subinoculation
1	180 ml I. V. from naturally infected calf	36 hours	<u>B. bigemina</u>	37½ hours
2	12 ml I.V. from No. 1	30 hours	<u>B. bigemina</u>	31 hours
3	12 ml I.V. from No. 2	24 hours	<u>B. bigemina</u>	24 hours
4	12 ml I.V. from No. 3	37½ hours	<u>B. bigemina</u>	37½ hours
5	12 ml I.V. from No. 4	57½ hours	<u>B. bigemina</u>	-----

## SUMMARY

Experiments were carried out to separate B. bigemina from B. argentina, B. major and A. marginale. The method of separation was rapid passage through 5 splenectomized calves. Five blood passages were carried out in 6-1/2 days. Babesia argentina, B. major, and A. marginale were eliminated as contaminants after 4 passages. A frozen stablitate of the isolated B. bigemina was established.

A Method for the preparation and examination of combination thin and thick blood films for the detection of Babesia parasitemia was developed. The technique for the staining of the combination thin and thick films involved the use of a phosphate buffered Giemsa stain solution containing alkyl phenoxy polyethoxy ethanol. Babesial CF antigens were also prepared and titrated for use in a CF microtiter procedure.

Babesia bigemina parasitized blood exposed to varied doses of gamma radiation up to 60 KRad was inoculated into calves. Calves infected with  $1 \times 10^{10}$  B. bigemina parasitized erythrocytes exposed to doses up to and including 30 KRad developed progressive parasitemias. Some calves receiving  $1 \times 10^{10}$  parasitized erythrocytes irradiated at levels of 36 and 42 KRad did not develop progressive infections. Progressive infections were prevented by exposure to irradiation at 48 KRad or higher. Subinoculation into susceptible splenectomized calves from parasites thus treated failed to produce active infections.

In addition to a lower infection rate brought about by irradiated parasites, calves that did become infected had prolonged prepatent periods and lower maximum parasitemia. Control calves, however, that had been inoculated with  $1 \times 10^7$  nonirradiated parasitized erythrocytes also had prolonged prepatent periods and lower maximum parasitemias. Therefore, the prolonged prepatent periods and lower maximum parasitemias in calves that had received  $1 \times 10^{10}$  irradiated B. bigemina could have been due in part to a reduction in the number of viable parasites injected. The finding that calves inoculated with  $1 \times 10^7$  nonirradiated parasitized erythrocytes had prolonged prepatent periods and lower maximum parasitemias indicate that such a standardized inoculum might be of use as a vaccine to produce an attenuated infection.

A degree of acquired resistance to infection with B. bigemina developed in calves after 1 inoculation with B. bigemina parasitized blood irradiated at 48 and 60 KRad. The resistance was sufficient to suppress multiplication of the Babesia and to permit calves to survive otherwise severe clinical infections with nonirradiated parasites. There was also less erythrocytic destruction and a smaller increase in rectal temperatures following challenge. Presumably the irradiated parasites were responsible for the development of resistance since irradiated nonparasitized blood did not produce a discernable acquired resistance. The observation that inoculation of calves with irradiated B. bigemina stimulated a degree of protective immunity, suggests that the

presence of replicating Babesia in the host is not necessary for the development of acquired resistance.

A similar degree of acquired resistance to infection with B. bigemina developed in calves inoculated with  $1 \times 10^{10}$  B. bigemina irradiated at 48 and 60 KRad and in calves inoculated with  $1 \times 10^{10}$  nonirradiated B. bigemina. It seems likely that the protective immunity produced with irradiated B. bigemina may be similar to that produced with living pathogenic B. bigemina in non-fatal infections. The acquired resistance to infection with B. bigemina developed in calves inoculated with  $1 \times 10^{10}$  B. bigemina irradiated at 48 and 60 KRad was much greater than the acquired resistance to infection developed in calves inoculated with  $1 \times 10^{10}$  heat killed B. bigemina. Thus, it seems likely that immunization with irradiated nonreplicating Babesia may provide the special immunological properties of living parasites important for producing a strong immunity while suppressing the pathogenic effects of the parasite.

The success obtained in immunizing calves with B. bigemina which had been irradiated, frozen, and stored in a dry ice cabinet may be of practical significance in future studies. The Babesia parasites could be irradiated and frozen, without loss of immunizing properties, for use in distant parts of the world.

DEVELOPMENT OF A RAPID CARD AGGLUTINATION TEST FOR DIAGNOSIS OF BOVINE  
BABESIOSIS.

INTRODUCTION

Babesia bigemina and Babesia argentina antigens were isolated from infected erythrocytes and are routinely used in complement fixation (CF) test in our laboratory. The CF test is tedious to perform and considerable technical knowledge, laboratory equipment and skill are required. Therefore, there is an evident need for a rapid field test for babesiosis which can be adapted for field conditions for detection of specific Babesia antibodies in cattle. A method of preparing an agglutinating antigen for use in a minute card test that may serve as a field screening test for babesiosis is described in this report:

MATERIALS AND METHODS.

Babesia bigemina (Montería isolate) was used for infecting calves and for producing B. bigemina antigens. Babesia - antigen was prepared from blood in which at least 20% of erythrocytes were parasitized. High parasitemias were developed by serial passage of the parasites through 2 to 3 splenectomized calves. In order to enhance development of parasitemias Vetalog (Corticosteroid hormon) 30 mg per day was injected IM, three days prior infection. During the course of each clinical attack following blood passage, thin and thick blood films of peripheral blood were taken every 2 to 3 hours to determine the portion of parasitized erythrocytes. When the parasitemia remained constant, as indicated by little or no change of the successive examinations, the organisms were passaged to the next animal. After the second

or third passage with Babesia bigemina up to 25% of erythrocytes were parasitized.

Blood for the preparation of antigen was drawn into flasks containing ethylene diamine tetraacetic acid (EDTA), 1.2 g/liter from calves with 25% parasitemia. Plasma was removed by centrifugation (Sorvall centrifuge) 2,500 rpm for 30 minutes. The infected erythrocytes were washed twice in 0.85% NaCl. The last wash was in antibiotic - saline solution containing 100 units of Penicillin and 100 mg of Streptomycin (Gibco, Biological Co., Grad., Island, N.Y. 14.072). Then, ten volumes of sterile 0.35% NaCl was used to lyse the infected erythrocytes for 1 hour at 4°C. The lysate was centrifuged at 2,500 rpm for 30 minutes, and resuspended in antibiotic-saline solution. Thirty-five ml of Babesia - suspensions were passed through the French Pressure Cell (American Instrument Co., Silver Spring), at 1,200 P.S.I. Then, the material was centrifuged at 10,000 g at 3°C for 30 minutes. The supernatant fluid was discarded and the concentrated parasites were resuspended in equal amounts of sterile antibiotic-saline solution. The suspension was homogenized in tissue grinders (Ten-Brock). One percent of Fast Green Dye (National Aniline Division, Co.) was added at the rate of 1 ml/25 ml of parasite suspension, mixed by shaking, and the mixture passed through the French Pressure Cell at 1,200 P.S.I. Excess dye was removed from the antigen by decanting the supernatant fluid after centrifugation at 10,000Xg for 30 minutes. The antigen pelet was resuspended in equal amount of antibiotic saline solution and homogenized in Ten-Brock grinder and sonified in Cell Disruptor, (Heat, Systems-Ultrasonics, N.Y.) for 3 minutes at 60 cycle power source, and tested for antigenic activity in a plate test. Serial dilutions of antigen was made and a approx. 0.025 ml of antigen was mixed with the equal amount of serially diluted sera.

Reference samples of serum for evaluating Babesia - antigen reactivity and stability were obtained from 3 calves in the carrier phase of Babesia infection and from two normal calves originated from area free of babesiosis. After mixing the antigen and serum, the tray was kept at room temperature 22-24°C. for reading immediately and from one to 60 minutes thereafter. The test was performed in a manner similar to technique used in the plate test for brucellosis. The use of disposable plastic trays to perform the test was found satisfactory.

### RESULTS

Lysis of Babesia bigemina infected erythrocytes with sterile 0.35% NaCl yielded an antigen composed of concentrated Babesia bigemina parasites. After passage through the French Pressure Cell the Babesia - parasites were ruptured and the unstained Babesia - antigen was grayish white and free of visible hemoglobin. Fine small particles of Babesia parasites were stained with Fast Green dye and this mixture was stable and uniform.

Preliminary studies with this antigen revealed that degree of specific agglutination reaction occurred with the serum from Babesia infected animals. This reaction was visible in period of 1 to 3 minutes, characterized with agglutination clumps which persisted during 24 hours. However, non specific agglutination occurred in a low serum dilutions. Results were encouraging and methods of eliminating of inherited problems of specificity of the reaction are being studied. In adjusting the agglutination reaction to detect a low antibody levels care had to be taken that antigen is not overly sensitive causing it to react with serum samples from uninfected cattle.

F I N A L       R E P O R T

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Subject : Visiting tropical zones in Perú (Instituto Veterinario de Investigaciones Tropicales y de Altura), June 25 - August 3; 1971.

Purpose : Helping to organize the necessary research for control of hemotropic diseases in Perú

Sponsor: Agency for International Development - United States AID Mission to Perú.



FINAL REPORT OF THE VISIT OF DR. R. TODOROVIC IN PERU,  
JUNE 25 TO AUGUST 3, 1971

SUMMARY

This report is a summary of all the activities of Dr. R. Todorovic in Perú from June 25 to August 3, 1971. The object of this visit was to help in organizing necessary investigations for the control of diseases produced by hemoparasites in bovine blood. During the visit it was observed that a great potential existed for beef and dairy cattle in tropical (Selva-Pucallpa) and sub-tropical (Oxapampa) zones of Perú, however, one limiting factor consists of the existence of hemotropic diseases (anaplasmosis and babesiosis) and their vectors. The following report describes the problem of hemoparasites infections in Perú, studies program, observations, conclusions, and recommendations for future work in this area.

INTRODUCTION

Dr. R. Todorovic arrived at Lima, Perú, on June 25th, by invitation of the United States AID Mission in Perú, for projecting an evaluation program for control of the hemotropic diseases in cattle. This invitation was solicited by the Director of the Institute of Tropical Veterinary Medicine of the National University of San Marcos, Dr. M. Moro. Dr. Todorovic was invited to assist in evaluating the hemotropic diseases problem in Perú and to give necessary recommendations for future investigations. He worked in cooperation with the National University professors Drs. Castillo, Guerrero, Caletti, Alvarado and Carpio, and also with the officers of the Ministry of Agriculture and Public Health. After evaluating hemotropic diseases problem in Perú this group discussed the means to develop an effective program for the control of bovine babesiosis and anaplasmosis. This was the second visit to Perú, the first being in May, 1970, for the Third National Congress of Veterinary

Medicine and Animal Sciences where the author of this report presented a work about research and control of hemotropic infections in Colombia, South America. During this first visit he had the opportunity to discuss problems relating to hemotropic infections with Peruvian investigators and he also taught a course: "Bovine Babesiosis" for the veterinarians of this country. All of this gave him intimate knowledge of the problems of hemotropic diseases of cattle in Perú.

#### STUDIES PROGRAM

June 26. Dr. Todorovic arrived to Lima.

June 28. In the morning, discussion in the US-AID offices in Lima with Mr. Curry Brookshier and Mr. R. G. Cason, F & A officers and others, about the program and objects of the visit. In the afternoon, a visit to the Institute of Tropical Veterinary Medicine to meet the Director, Dr. Manuel Moro and the Dr. Patrick Guilbride representative of FAO and the professors Drs. Castillo, E. Caletti and C. Guerrero. A program for the visit was then arranged. The plan included a visit to the North Coast, the jungle highlands (Oxapampa) and lowlands (Selva-Pucallpa) and conferences with students, professors and professionals, about hemoparasitic diseases.

June 29 and 30. A visit to the National University - San Marcos, the College of Veterinary Medicine to meet the Director and Dean, Dr. Gustavo Ayllón and the professors of the different departments of Parasitology (Dr. Guerrero), Microbiology (Dr. Caletti) and Clinical Pathology (Dr. Castillo).

July 1. At the National University - San Marcos, College of Veterinary Medicine an invitation to teach a class in Parasitology on the subject Bovine babesiosis with special emphasis on etiology, life cycle of hemoparasites, pathogenesis of the disease, symptoms, lesions in bovines, diagnosis and immunology of Babesia bigemina and Babesia argentina and life cycle of the tick vectors; epidemiology, curative and prophylactic treatment and disease control. This course was organized for the third year students of College of Veterinary Medicine. The class consisted of thirty students with members of the Department of Veterinary Parasitology.

July 2. Invitation by the University Faculty of Veterinary Medicine to present a conference entitled "Diagnosis of Bovine Babesiosis - Serological Tests", which was attended by more than 80 persons including professors, students and veterinarians.

July 3 - 5. Participation in discussions on the problems and programs of hemoparasites with the National University - San Marcos professors, especially with Dr. Castillo and his assistants, and Drs. Carlos Guerrero and Eddo Caletti also. Afterward a visit to the Ministry of Agriculture, Dr. Teodorico Terry and the Ministry of Public Health, Dr. Cesar Lora was made, in order to discuss the problem of hemoparasites in the Peruvian Jungle (Selva-Pucallpa). Information was received of the results of prior investigations in Perú with hemoparasites, and also of the plans for the livestock industry development in the future in Perú.

July 6, 7, 8. Trip to the North Coast of Perú, Piura, in order to observe and evaluate the problem of the hemoparasites in that region; accompanied by Dr. Augusto Castillo and Dr. Carlos Guerrero. During this period they met Dr. P. Regalado, of the Substation of Tropical Veterinary Medicine in Piura; Dr. N. Vasallo, from the Cattlemen's Association in Piura, and Dr. Adan Vasquez, from the Ministry of Agriculture. A visit was made to the farms which had problems with hemoparasites (and was sending animals to the Pucallpa-Jungle). Accompanied Drs. Castillo and Guerrero to the Sol-Sol Farm, Ganadera Bajen Farms, 70 kms. from Piura, that have imported cattle Santa Gertrudis breed from the USA, Texas, and Criollos breed. The next visit was to the farm "La Gopa", at which the major breed of cattle are Santa Gertrudis imported from Texas. This farm is very close to the frontier of Ecuador and approximately 123 kms. from Piura. Around Piura they visited two dairies with Brown Swiss and Holstein breeds, and finally they visited the farm Pabur Oria, in Potrerillo which has imported cattle, from Brazil, and Criollo cattle, and the Mr. Romero Mallares Farm with Brown Swiss dairy cattle. This farm has been affected by the Agrarian Reform, and made a Cooperative.

In total approximately 2,500 to 3,000 animals of Santa Gertrudis, Brown Swiss, Holstein, Zebu and Charolais breeds were observed, the

majority of them imported from the United States, Brazil, Ecuador or Colombia. A representative sample consisting of blood and ticks was taken from 10% of the cattle. The blood was taken without coagulant for serological tests, and with anticoagulant for blood smears, hemoglobin determination and hematocrits. The samples were taken to determine the presence and prevalence and effects of anaplasmosis, babesiosis and trypanosomiasis. The ticks collected will be identified later. . .

The natural pastures were observed, with samples taken from different fields. By invitation of the Technical University of Piura, a conference was given on the topic "Aspects in the Control of Hemoparasites and their Vectors in the Field". The conference was attended by the veterinarians of Piura and Tumbes, the cattlemen of that region, professors, and students. After finishing there was a round table discussion, with the participation of Dr. Augusto Castillo and Dr. Carlos Guerrero, approximately 100 persons attended.

July 9, 10. Trip to Chiclayo, Lambayeque by invitation of Dr. Enrique Arbaiza, Dean of the National University "Pedro Ruiz Gallo". In Lambayeque, Drs. Todorovic, Castillo and Guerrero visited the dairies of the Ministry of Agriculture and University. The animals were of the Holstein breed, imported from the United States and Holland. A talk concerning cattle losses was given by Dr. Enrique Arbaiza of the National University and of the Veterinary College of Lambayeque. Dr. Todorovic presented a paper on the topic "Hemoparasites in Bovines, their Investigation and Control". A round table discussion was carried out with Dr. Castillo and Dr. Guerrero; there were 50 persons attending, including professionals, professors and students.

July 12 to 17: Trip to the Principle Tropical Station of the Institute of Tropical Veterinary Medicine in the Pucallpa Jungle and cattle ranches of San Jorge and Turnavista of the Ministry of Agriculture. Accompanying Dr. Todorovic, Dr. Augusto Castillo, Dr. Carlos Guerrero, Dr. Cesar Lora and Dr. Eddo Caletti, Dr. Manuel Moro, the director of the Institute of Tropical Veterinary Medicine and Dr. Miguel Alberto Cárdenas, economic Director of the University.

They were also accompanied by Dr. Cesar Lora of the Ministry of Public Health. They were received by Dr. Jorge Alvarado, chief of the Parasitology Laboratory of the Principal Tropical Station in Pucallpa. The object of this visit was to investigate what type of hemoparasites existed in the cattle of this region. For this reason 3 native animals were splenectomized. Blood samples were collected every day for a period of 3 to 4 weeks (afterward samples from approximately 10% of the cattle population were collected). The blood was collected without coagulant for serological tests, and with anticoagulant for tick and thin blood smears, to determine the presence of babesiosis and anaplasmosis and fresh blood smears for the presence of Trypanosoma. Inoculation of laboratory animals was also carried out. The blood samples were evaluated by hematocrit and hemoglobin determinations. Ticks and natural pasture were also collected for identification purposes.

The splenectomized animals demonstrated the presence of Babesia spp. 3 days after surgery and Anaplasma marginale a week after surgery. The blood from two splenectomized animals was collected and it was subinoculated into two intact animals to study the pathogenicity of the hemoparasites.

A visit was made to the Ranch San Jorge, which has a population of 3,000 animals including the Santa Gertrudis mentioned above from Piura and Charolais, Zebu and Criollos. Here ticks were collected along with the samples mentioned above. The ticks were identified as Boophilus microplus. The ranch Turnavista was also visited, located in the jungle 60 kms. from Pucallpa. The visit was made by light aircraft. This ranch has been bought by the Ministry of Agriculture, and has a population of approximately 2,000 animals, the majority being Zebu and Zebu crosses. The samples were collected in the same manner as described above. It is very interesting to emphasize that the ticks collected from the animals were identified as Boophilus microplus and for the first time Amblyomma spp.

Around Pucallpa, the Amazon Hospital Station with imported Brown Swiss and the Acosta Farm with Holsteins imported from Lima were visited.

In both, the collection of samples was the same as previously described. In the Acosta Ranch a high rate of infestation of Boophilus microplus and an infection of Anaplasma marginale were found; the animals were in very poor physical conditions. Finally, the group went to the Institute of Tropical Veterinary Medicine farm that has the dairy breeds Brown Swiss, Ojero Holandés (imported from Holland), Red Danish and cross breeds for breeding with beef cattle. Here samples were collected in the same manner as above.

The plan of work in the Principle Tropical Station Selva-Pucallpa consisted of collecting samples, visiting the ranches and farms in the morning and working in the laboratory in the afternoon. The last day of the visit to the Station a conference was given entitled "Some Aspects in the Control of Babesiosis in the Field", at which veterinarians of the region and the personnel of the Institute of Tropical Veterinary Medicine assisted, for an audience of approximately 50 persons. After ending the conference there was a round table discussion with Drs. Todorovic, Castillo, Guerrero, Jorge Alvarado and Caletti.

July 19 to 24. A visit to the laboratories of the Academic Program of the College of Veterinary Medicine, with emphasis in the laboratories of parasitology and clinical pathology. In these laboratories some laboratory animals were inoculated with blood of animals from the tropics (Pucallpa), in order to determine the presence of Trypanosoma infections.

July 22. A seminar was given to the Peruvian Society of Parasitology concerning "immunological Aspects of the Hemoparasites of Cattle". Around 25 professional parasitologists from different institutions attended (Ministry of Health, Universities, and Private Practitioners) After the presentation of the subject there was a discussion. All the participants agreed that the solution to the problem of hemoparasites in Perú was a system of premunition, because one cannot eradicate the vectors.

July 23. In the morning there was a meeting with the members of the Ministry of Agriculture, Drs. Emilio Matto, Director of Peruvian Agriculture Promotion; Teodorico Terry, Subdirector of Pecuaria Promotion; Carlos Narváez, Director of Quarantine; Engineer Jorge

López, expert in Pastures; and other members of the Ministry. Dr. Augusto Castillo, professor of the National University of San Marcos and member of Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA) assisted. The object of the meeting was to discuss the problems of control of hemoparasites in imported animals (the requirements to clean the animals in quarantine), and organizing a premunition program.

Members of the Ministry showed much interest in trypanosomiasis in respect of diagnosis, treatment, prophylaxis, epidemiology, and economic losses. They asked questions in relation to quarantine and showed much interest in the development of a control program for hemoparasites in Perú.

In the afternoon there was a meeting in the Institute of Tropical Veterinary Medicine with a final discussion about the observations of the different zones visited. Presiding were Drs. Augusto Castillo, Carlos Guerrero, Eddo Caletti, Jorge Alvarado and Todorovic. There was an exchange of opinions and discussion concerning hemoparasite investigations carried out by members of Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA). For example, the Department of Parasitology (Dr. Guerrero) is in charge of developing a program for the control of ticks. The Department of Microbiology (Dr. Caletti) for the development of a diagnostic system and the Clinical Pathology Department (Dr. Castillo), the development of a diagnostic and premunition programs.

At 4:30 in the afternoon, a conference at the National University - San Marcos was given on "Bovine Babesiosis and its Control" for professionals, interested professors, representatives of the Ministry of Agriculture and Ministry of Public Health, with 20 persons attending. After the conference there was a round table discussion.

July 25 to 28. A visit to the Oxapampa Zone was made, in order to observe animals, pastures, ranches and the existence of diseases produced by hemoparasites. Dr. Todorovic was accompanied by Drs. Augusto Castillo and Carlos Guerrero. They arrived at

Oxapampa at 7:00 pm. on July 25th and the same night there was a meeting with Dr. Alejandro Alva, Official Veterinarian of the Ministry of Agriculture, concerning a study program of hemoparasites.

July 26. At 8:00 am. a visit to the Ranch Huancabamba owned by Mr. Werner Müller, located 25 km. from Oxapampa, was carried out. On this ranch there are 500 Holstein - Zebu crossed animals. This ranch has two types of pasture; Melinis minutiflora (Gordura) and Pangola grass. The Gordura grows in the lowlands while Pangola grows in the highlands. There were few ticks. Some of them were collected and identified as Boophilus microplus. Blood samples were taken from the animals for serologic tests. Farmers were practicing dipping for tick control in the rainy season at irregular intervals. Purebred Holstein were seen, obtained from the National University of San Marcos, Faculty of Veterinary Medicine.

At 1:00 in the afternoon the Travi's Huancabamba Ranch was visited. This ranch is located near Müller's and has approximately 550 Purebred Santa Gertrudis and Criollo crosses and Brown Swiss. Six years ago they started eradicating ticks by dipping each 15 days for almost 2 years. For approximately 4 years this ranch was free of ticks and had been quarantined. This indicates that it's possible to eradicate ticks in zones where babesiosis is endemic and hence control the disease. At 3:30 pm. a visit to the ranch "La Esperanza" owned by Mr. Ernesto Müller was carried out. This ranch has Purebred Holsteins for cross breeding that will produce approximately 8 liters of milk without feeding concentrates, these animals only eat natural pasture. The animals had little tick infestation on this ranch. It is estimated that the potential for increasing the number of animals is from 12,000 to 14,000 and the hemoparasite losses may be controlled by a tick eradication system existing in this zone.

July 28 to August 2. A research program was discussed with the members of the Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA) to be applied in the Jungle (Pucallpa). A report of this visit and a meeting with the Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA) Staff board was made.

### Hemoparasites and their Impact on the Economy of Perú

The major economic problem resulting from the hemoparasites diseases in Perú is related to the introduction of cattle free from hemoparasites to the tick infested endemic areas. In practice, this problem is of primary importance in relation to imported cattle and the cattle of the highlands that are introduced to lowland pastures of the Jungle or the North Coastal areas.

Several reports, inquiries and scientific observations indicate that babesiosis and anaplasmosis are the most harmful diseases for the Peruvian livestock industry development. The first disease is caused by Babesia spp. and the second is produced by Anaplasma marginale. In the natural form, the infections are transmitted by blood biting arthropods. In Perú, there are several ticks species: Boophilus microplus and Amblyomma spp. They probably constitute the most important babesiosis and anaplasmosis vectors. There are no figures concerning the incidence of trypanosomiasis. The characteristic signs of the three hemotropic diseases when they are present alone or together are: progressive anemia, icterus, fever, anorexia and finally death. In addition hemoglobinuria is usually observed in babesiosis.

#### OBSERVATIONS

Based on results of the visit to the Peruvian North Coast and Jungle, one can say that in general there is a great potential for a beef and dairy cattle industry. However, one of the limiting factors are the diseases produced by the hemoparasites (A. marginale, Babesia spp.) and their vectors (principally Boophilus microplus).

1. During the visit made by Drs. Todorovic, Castillo, Guerrero, Caletti, and Lora to the North Coast zone (Piura) and the Jungle lowlands (Selva-Pucallpa) and Jungle highlands (Oxapampa) few animals in relation to the abundant natural pastures were found. In all the zones there were animals infected with Boophilus microplus, Anaplasma marginale and Babesia spp.

2. In some Jungle zones (Pucallpa) like Turnavista and others, the beef cattle population can be increased in order to utilize the existing pastures.
3. The Oxapampa Valley has conditions for the development of pasture for beef and milk cattle, having roads communication with Lima. In general, there are tick infestations but it was observed that one ranch (Huarcabamba) had eradicated ticks. The first generation Zebu-Holstein cross had shown an increase in meat and milk production higher than the Holstein-Criollo cross.

High tick and hemoparasite infestations in animals were not observed probably due two reasons: a) The effect of the Gordura grass; and b) the effect of cross breeding. The Gordura grass grows easily and abundantly and the animals readily consume it, but it does not exist on a 100% of the farms. The ranchers do not have information about its effect on ticks, however, a low tick infestation was observed in this area.

4. In North Coast zones there are imported purebreed cattle that have become acclimatized and could be used for beef cattle development.
5. Animals naturally premunized and moved from the North Coast (Piura) to the Jungle (Pucallpa) became clinically infected with hemoparasites. It is probable that different strains of Babesia spp. or Anaplasma marginale exist.
6. A system of organization for the diagnosis and control of hemoparasites is needed.
7. In some zones acaricides (chemical) in appropriate forms were not used; consequently, a great deal of infestation was found (Acosta's Ranch, Selva-Pucallpa).
8. There is scarcity of animals, installations and adequate equipment in the places visited to investigate hemoparasites and their vectors.

9. Coordination among investigators from different disciplines is needed. Rather than acknowledging these investigators personally, Dr. Todorovic acknowledges their efforts, scientific knowledge, vigor, enthusiasm and determination to do something constructive about the disease problems in their country.

#### CONCLUSIONS

1. In the zones visited Piura (North Coast), Pucallpa (Lowland Jungle) and Oxapampa (Highland Jungle), the presence of Babesia spp. and Anaplasma marginale in Bovines was demonstrated.
2. In the areas visited different grades of the principal vector of these hemoparasites infestation, Boophilus microplus, were found.
3. In addition to Boophilus microplus, Amblyomma spp. was demonstrated in the Pucallpa (Turnavista region).
4. The presence of many ticks in some jungle livestock herds leads to poor physical condition of livestock due to the blood loss by tick feeding as well as disease transmission.
5. In the Oxapampa (Highland Jungle) Melinis minutiflora exists. The Boophilus microplus infestation is low and in some areas is eradicated. Anaplasmosis and babesiosis are controlled easily, by efforts in tick control and disease treatment. In the zone (Oxapampa) a new Zebu and Holstein cross is being raised which promises good results in both beef and dairy cattle production in the tropical zone.
6. The naturally premunized Piura animals which were carried to Pucallpa (Lowland Jungle) were clinically infected with hemoparasites. This indicates loss of immunity or differences in strains of hemoparasites.
7. A good system for hemoparasite serological diagnosis does not exist in the laboratories visited.

8. In the Pucallpa and Turnavista (Lowland Jungle) zones small areas with Melinis minutiflora (Gordura grass) were found and it is believed it has a dilaterous effect on the tick larvae.
9. Upon a direct examination of fresh or stained blood smears, Trypanosoma spp. were not detected. However, the inoculation of splenectomized and laboratory animals follow in observation.
10. It is necessary to develop a program for the control of hemo- parasites and their vectors before large investments are made.

#### RECOMMENDATIONS

It is very important to form a group of investigators interested in working with hemoparasites to discuss and analyse all research projects to be considered. It will be necessary to include in this group members in different fields such as parasitology, immunology, clinical pathology, microbiology, etc.

1. To establish a project for the study and control of hemoparasites and their vectors. Consequently, this would bring an increase in the dairy and beefcattle production in tropical zones.
2. Basically, the project should consider the following aspects:
  - a) To establish coordination between different groups of participant investigators (parasitologist, microbiologist, pathologist, etc.).
  - b) To provide sufficient funds for developing and performing the project (material and equipment).
  - c) To establish contacts with international organizations for working in these aspects in order to interchange information, materials and investigators.

3. Within the project the following aspects must be considered:
- a) Development of serological tests for hemoparasites diagnosis.
  - b) Prevention method studies based on the vaccination and standardized premunition with field strains. Pastures improvement and control of the tick population especially in lowland areas.
  - c) Prophylactic and curative drugs experimentation.
  - d) A control of vector (ticks) systems investigation.

#### ACKNOWLEDGEMENT

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A CLINICAL, SEROLOGICAL AND PATHOLOGICAL STUDY OF CONCURRENT ANAPLASMOSIS  
AND BABESIOSIS IN EXPERIMENTALLY INFECTED CALVES

INTRODUCTION

Anaplasmosis and babesiosis are infectious, transmissible diseases caused by hemoparasites. Although several domestic and wild animal species are susceptible to the infection caused by Anaplasma and Babesia organisms, economic losses are most severe in cattle. The incidence of bovine anaplasmosis and babesiosis is especially high in the tropical and subtropical regions of the world where large enzootic areas exist. Indigenous cattle within the enzootic areas are normally exposed to infection at an early age when their resistance to the disease is reported to be highest. The indigenous cattle usually display minimal signs of a clinical infection and subsequently develop a chronic carrier state or infection through a process of natural premunition. However, cattle which are transported from non-enzootic areas into or through enzootic areas for the purpose of shipment to market, utilization of pasture land or as imported breeding stock are highly susceptible to severe clinical infection with high mortality rates often occurring.

Numerous investigators have commented on the common distribution of anaplasmosis and babesiosis and on the high frequency of concurrent Anaplasma and Babesia infection of cattle. Several investigators have observed and increased severity in clinical illness in cattle concurrently infected. Others have reported the results of experiments which indicate that either of the disease agents may initiate in the bovine host a physiological state which is not conducive to the multiplication of the other disease agent.

The purpose of this investigation was to conduct an experiment especially designed to study the clinical course, serological response and pathological manifestations of cattle concurrently infected with Anaplasma marginale and Babesia bigemina; and further, to study the biological behavior of the 2 disease agents during a concurrent infection.

## MATERIALS AND METHODS

## Experimental Organisms

Isolation. Pure isolates of Anaplasma marginale and of Babesia bigemina used during the experiment were obtained by natural Boophilus microplus transmission of the organisms to susceptible calves under field conditions. Two intact and 2 splenectomized Holstein-Freisian calves, 4 months of age, with a known history of no exposure to tick vectors, negative complement fixation tests for Anaplasma and for Babesia and with negative blood smears for hemoparasites were transported by airplane from the savanna of Bogotá to the North Coast of Colombia and placed in tick infested pastures at the Turipaná ICA experimental station near Montería, Córdoba. Daily blood samples were collected from the calves, and packed cell volumes and body temperatures were ascertained. Giemsa stained blood smears were examined daily for the presence of hemoparasites.

Babesia bigemina. Following 18 days exposure to tick vectors, one of the splenectomized calves was observed to be infected with B. bigemina and B. argentina from stained blood smears. One hundred ml of blood was collected from the calf using ethylene diamine tetra acetic acid (EDTA) disodium salt, 2 mg/ml of blood as an anticoagulant, transported to Bogotá and immediately upon arrival inoculated intravenously into a splenectomized calf. The separation of B. bigemina from B. argentina and from A. marginale was accomplished by the rapid serial passage of infected blood through 5 splenectomized calves following the method described by Callow and Hoyte.<sup>9</sup> Blood was collected from the last calf

of the rapid transfer series and inoculated into 2 intact 6 month old Holstein-Freisian calves. The last 2 calves of the rapid transfer series were treated intravenously with trypan blue<sup>a</sup> to prevent death from acute babesiosis due to infection with B. bigemina to allow the continued observation of the calves for infection with B. argentina. Packed cell volumes and Giemsa stained blood smears collected from the 2 splenectomized calves and from the 2 intact carrier calves were examined twice weekly for 12 weeks following inoculation to ascertain the purity of the B. bigemina isolate and the absence of other hemoparasites.

Anaplasma marginale. A mixed infection of A. marginale, B. bigemina and B. argentina was observed in an intact calf at the ICA experimental station 32 days after exposure to tick vectors. One hundred ml of blood was collected from the calf using EDTA as an anticoagulant, transported to Bogotá and immediately upon arrival inoculated intravenously into a splenectomized calf. The splenectomized calf was treated intramuscularly at the time of inoculation and for 2 consecutive days thereafter, with 4-4', diamidino diazoaminobenzene diacetate<sup>b</sup> at a dosage of 3,5 mg/kg of body weight<sup>3</sup>. Packed cell volume and Giemsa stained blood smears were examined from the calf 3 times weekly for 6 weeks following inoculation to observe the clinical progression of anaplasmosis and to ensure that the Babesia were eliminated by chemotherapy.

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a. Azul Tripan: Velox Laboratories, Bogotá, Colombia

b. Ganaseg: E. R. Squibb & Sons, Interamerican Corporation, Cali, Colombia.

Preservation and Storage of Babesia Bigemina. Inocula containing B.

bigemina were prepared from the blood of a splenectomized calf having a pure patent infection. Immediately prior to preparation of the inocula, the calf was observed to have an erythrocyte count of  $8.5 \times 10^6$  erythrocytes/mm<sup>3</sup> of blood, with 2.5% of the erythrocytes being infected with B. bigemina. Each ml of blood from the calf was calculated to contain approximately  $2.1 \times 10^8$  B. bigemina. Five ml of blood was aseptically collected from the calf into each 7 ml evacuated glass tube<sup>c</sup> using EDTA 2mg/ml of blood as an anticoagulant. Following the collection of blood, sterile 95.8% glycerol<sup>d</sup> was added to each tube as a cryoprotectant to produce a final glycerol concentration of 10.7% by volume. The blood and glycerol were mixed by inverting the tubes several times and the tubes placed in a refrigerator at 4 C. Following 2½ hours of refrigeration, the tubes were removed from the refrigerator, and the blood and glycerol mixture was frozen by plunging the tubes into a solid carbon dioxide -absolute alcohol bath. The tubes were immediately placed in a dry ice chest at -79 C and stored until used as inocula.

Preservation and Storage of Anaplasma marginale. Inocula containing Anaplasma marginale were prepared from the blood of a splenectomized calf having a pure patent infection. Immediately prior to preparation of the inocula, the calf was observed to have an erythrocyte count of  $5.5 \times 10^6$  erythrocytes/mm<sup>3</sup> of blood with 3.5% of the erythrocytes being infected with A. marginale. Each ml of blood from the calf was calculated to contain approximately  $1.9 \times 10^8$  A. marginale. The inocula were prepared from the blood of the calf using a modification of the method reported by Barnett.<sup>4</sup> Blood was aseptically withdrawn from

<sup>c</sup> B.D. Vacutainers: Becton-Dickenson and Company, Rutherford, N.J.

<sup>d</sup> J.T. Baker Chemical Company, Phillipsburg, N.J.

the calf and collected in a 250 ml sterile flask using heparin<sup>e</sup> 4 units/ml of blood as an anticoagulant. Modifications of Barrett's method included: the addition of sterile 95.8% glycerol to the blood to produce a final concentration by volume of 8.2% glycerol; five ml aliquots of the blood and glycerol mixture were dispensed into 7 ml glass tubes and the tubes sealed with rubber stoppers; temperature decrease during the freezing process was 1°C per minute to -16°C, at which time the temperature was lowered to -79°C over a 5 minute period. The tubes of frozen blood were placed in a dry ice chest at -79°C and stored ~~there~~ until used as inocula.

#### Experimental Animals

Twenty-two, male, nonsplenectomized, Holstein-Friesian calves, 7 months of age were used as experimental animals. Each calf was identified by an ear tag number and data collected during the experiment was referred to by these numbers. The calves were housed in a tick free environment prior to and during the course of the experiment. Insect control was maintained by the periodic spraying of the housing facilities with an appropriate insecticide.<sup>f</sup> A balanced ration of ensilage and a concentrate mixture composed of 43% corn, 30% cotton seed oil meal, 24% bran, 1% salt, 1% bone meal and 1% trace minerals was provided to the calves during the experiment. Water and salt were provided free choice. Fecal specimens were collected from each calf prior to experimental infection and examined for helminth and protozoan parasites and treatment was deemed to be unnecessary.

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<sup>e</sup> Heparin (Ammonium salt): Distributed by Scientific Products Division, American Hospital Supply Corporation. General Offices Evanston, Ill.

<sup>f</sup> Vapona: O-O dimetil 1-2-2 diclorovinil fosfato, Shell Ltda., Bogota, Colombia

Prior to experimental infection, all calves were screened for the presence of current or previous infection with Anaplasma or with Babesia parasites by the complement fixation test,<sup>2,82</sup> by the examination of stained blood smears and by the inoculation of a splenectomized calf with a pooled blood sample composed of 10 ml of blood collected from each experimental calf. Blood samples were collected weekly from the splenectomized calf for 12 weeks following inoculation of the pooled blood sample, and packed cell volumes and stained blood smears were examined to ascertain the absence of hemoparasites.

The calves were examined physically, and blood and serum samples were collected twice weekly for 2 weeks prior to experimental infection to ascertain normal values for the following parameters: packed cell volume; hemoglobin concentration; total and differential leukocyte counts; reticulocyte count; total, direct, and indirect serum bilirubin concentration; serum glutamic-oxalacetic transaminase activity; serum sorbitol dehydrogenase activity; total serum protein content; serum protein electrophoretic patterns; body weights; and body temperature.

Following the collection of blood and serum samples necessary to ascertain preinfection values for the above mentioned parameters, the experimental calves were randomly divided into 4 groups. Group I and II each contained 3 calves, while Groups III and IV each contained 8 calves.

Inoculation of Experimental Calves. Immediately prior to the inoculation of the experimental calves, the tubes of frozen infected blood were removed from the dry ice chest and thawed by immersing the tubes in a water bath at 38 C for 1 minute. The calves were inoculated within 30 minutes after thawing the frozen inocula.

The 3 calves of Group I were each subcutaneously injected with 5 ml of thawed inoculum containing approximately  $9.5 \times 10^8$  A. marginale.

Group I served as a control to evaluate the response of calves singularly infected with A. marginale.

The 3 calves of Group II were each subcutaneously injected with 5 ml of thawed inoculum containing approximately  $10.5 \times 10^8$  B. bigemina. Group II served as a control to evaluate the response of calves singularly infected with B. bigemina.

The 8 calves of Group III were each subcutaneously injected with 5 ml of thawed inoculum containing approximately  $9.5 \times 10^8$  A. marginale. Seventy days following the inoculation of Group III, the calves had recovered from the acute clinical stage of anaplasmosis and were entering the carrier or post patent period as evidenced by a continual increase in packed cell volume and a continual decrease in the number of infected erythrocytes. The calves of Group III were each subcutaneously injected on day 70 with 5 ml of thawed inoculum containing approximately  $10.5 \times 10^8$  B. bigemina. Group III served to evaluate the effect of a secondary infection with B. bigemina in calves recently recovered from anaplasmosis.

The calves of Group IV were each subcutaneously injected with 5 ml of thawed inoculum containing approximately  $10.5 \times 10^8$  B. bigemina. Thirty-seven days following the inoculation of Group IV the calves had recovered from the acute clinical stage of babesiosis as evidenced by a continual increase in packed cell volume and a continual decrease in the number of infected erythrocytes. The calves of Group IV were each subcutaneously injected on day 37 with 5 ml of thawed inoculum containing approximately  $9.5 \times 10^8$  A. marginale. Group IV served to evaluate the effect of a secondary concurrent infection with A. marginale in calves recently recovered from babesiosis.

### Experimental Procedures

Sample Collection Schedule and Procedures. Blood and serum samples were collected twice weekly from each calf for the duration of the investigation. This schedule was altered during the acute clinical phases of anaplasmosis and babesiosis during which time samples were collected 3 times weekly. Samples were collected from the calves of all 4 groups until day 120 post infection.

Samples were collected between 8:00 and 10:00 a.m. Body temperature, in degrees centigrade, was recorded during each collection period, and the general attitude and condition of each calf were noted. Blood and serum samples were aseptically collected from the calves by intravenous puncture of the jugular vein. A total of 45 ml of blood was withdrawn from each calf at each collection. Five ml of the blood was placed in a 10 ml glass tube with EDTA, 2mg/ml of blood as an anticoagulant. The remaining 40 ml of blood was dispensed into 10 ml glass tubes. The blood was allowed to clot at 24 C and the serum collected following centrifugation at 2500 rpm for 15 minutes.

The body weight of each calf was approximated by tape measurement<sup>g</sup> prior to experimental infection and every seventh day thereafter. Changes in body weights were compared between the 4 groups of calves.

Bone marrow biopsies were taken from 2 calves randomly selected from each of the 4 experimental groups prior to experimental infection, and myeloid:erythroid ratios determined to approximate normal hematopoietic activity. Following experimental infection, bone

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<sup>g</sup> Metric tape for milk breeds c3115, NASCO, Fort Atkinson, Wis.

marrow biopsies were collected from the 2 calves of Group I and the 2 calves for Group II on the day following maximum parasitemia for Anaplasma and Babesia respectively. Bone marrow biopsies were taken from the 2 concurrently infected calves of Group III and the 2 concurrently infected calves of Group IV on the day following maximum parasitemia of Babesia and Anaplasma respectively. Bone marrow biopsies were again taken from the 2 calves of each group at the termination of the experiment. All bone marrow specimens were collected from the medullary cavity of the rib using the biopsy technique described by Coles.<sup>12</sup>

Gross and Microscopic Pathology. Pathological changes were evaluated by gross and by histopathological examinations. Four calves, 2 from Group III and 2 from Group IV, died during the experiment. A necropsy was performed on each of the calves and a complete set of tissue specimens were collected for histopathological examination. In addition, 2 concurrently infected calves from each of Groups III and IV were euthanatized, by electrocution using 110 volt alternating current, for necropsy on the day following maximum parasitemia of Babesia and Anaplasma respectively. Tissue specimens were collected from grossly normal and pathologic organs. The tissues were fixed in buffered 10% formalin, embedded in paraffin, sectioned at 5 microns, stained with hematoxylin and eosin and examined for microscopic abnormalities. In addition, special stains were used in the preparation of selected tissue specimens for histopathological examination. Special attention was given to the tissues of the reticuloendothelial system during histopathological examination, however, tissues from all body systems were examined. A normal, noninfected control calf was also euthanatized and subjected to necropsy. A complete set of tissue specimens were collected and utilized for comparative studies.

Clinical Pathology. The techniques performed on blood samples were completed on the same day as sample collection. Techniques analyzing serum samples were performed on samples which had been frozen and stored at -10 C.

Packed cell volumes were determined by the microhematocrit method.<sup>72</sup> Hemoglobin concentration was measured by the Hycel cyanomethemoglobin technique.<sup>h</sup> Differential and total leukocyte counts were determined by standard laboratory techniques.<sup>6</sup> Reticulocyte counts were conducted using a standard reported technique, utilizing brilliant cresyl blue stain, followed by counter staining with Wright's stain.<sup>72</sup>

The percentage of parasitized erythrocytes was determined by the microscopic examination of thin blood smears under the oil immersion objective. Following fixation in methyl alcohol, blood smears were stained for 20 minutes with a 1:20 stock Giemsa-buffered distilled water mixture at a ph of 7.2. A wetting agent<sup>i</sup> was added to the distilled buffered water to a final concentration of 0.01% to aid in removal of precipitated stain from the blood smears. The stained blood smears were washed in distilled water and dried under a heat lamp. The percentage of parasitemia was determined by counting from 10,000 to 12,000 erythrocytes on each slide. All counts were made by counting cells located from  $\frac{1}{2}$  to  $\frac{1}{4}$  inch from the feathered end of the blood smear. Thick blood smears were used for the detection of Babesia parasites in the blood of chronically infected calves.<sup>45</sup>

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<sup>h</sup> Hycel, Houston, Texas

<sup>i</sup> Triton X-100, Rehm and Haas, Philadelphia, Penn.

Total serum protein levels were ascertained by using a hand-held, temperature compensated refractometer.<sup>j</sup> Serum glutamic oxaloacetic transaminase levels were measured by a kit procedure<sup>k</sup> modified from the original method of Reitman and Frankel.<sup>64</sup> Serum sorbitol dehydrogenase activity was measured by a modified kit procedure<sup>l</sup> adopted from the original method of Gerlach.<sup>22</sup> Serum bilirubin concentration was determined by a modification of the Van den Bergh procedure as described by Gibson and Goodrich.<sup>23</sup>

Serum protein electrophoretic patterns were ascertained by a modification of the technique described by Briere and Mull using cellulose acetate membranes.<sup>8</sup> Five lambdas of serum were applied to cellulose acetate membranes<sup>m</sup> with a Titan M-2 applicator.<sup>n</sup> The cellulose membranes were presoaked in cold trisbarbitol -sodium barbitol buffer<sup>o</sup> having a ph of 8.8 and an ionic strength of 0.045 molar. Following application of the serum samples, the membranes were immediately transferred to a electrophoresis chamber<sup>p</sup>. Separation of the serum protein fractions was accomplished by applying a constant voltage of 300 volts or a constant amperage of 1.25 milliamperes to the chamber for 45 minutes. Following electrophoretic separation, the membranes were immediately placed in a solution of 0.5% Ponceau<sup>q</sup> with 100 ml of trichloroacetic acid and fixed-stained for 5 minutes. Excess and background stain was removed by rinsing the membranes for one minute in each of four 5% acetic acid baths. The membranes were then dehydrated by immersion for 30 to 60 seconds in absolute methyl alcohol

<sup>j</sup> T-S Meter, American Optical Company, Buffalo, N.Y.

<sup>k</sup> Sigma Chemical Company, St. Louis, Mo.

<sup>l</sup> Sigma Chemical Company, St. Louis, Mo.

<sup>m</sup> Sepraphore III: Gelman Instrument Company, Ann Arbor, Mich.

<sup>n</sup> Helena Laboratories, Allen Park, Miss.

<sup>o</sup> High Resolution Buffer, 51104, Gelman Instrument Company, Ann Arbor, Mich.

<sup>p</sup> Deluxe Electrophoresis Chamber 51170, Gelman Instrument Company, Ann Arbor, Mich.

<sup>q</sup> Gelman Instrument Company, Ann Arbor, Mich.

and cleared by immersion in a solution of 10% acetic acid in methyl alcohol. Following 20 minutes of drying at 55 C the membranes became transparent. The various serum protein fractions on the transparent membranes were relatively quantitated by using a densitometer with an automatic scanner and intergrator.<sup>r</sup> Absolute values for the various protein fractions were obtained from the relative percentages of each fraction and the total serum protein values.

Serology. Serum samples collected from each experimental calf were screened for the presence of complement fixing antibodies for A. marginale and for B. bigemina. All samples were screened for the presence of A. marginale antibody by the standardized complement fixation test for anaplasmosis as prescribed by the United States Department of Agriculture.<sup>2</sup> The complement fixation test employed to detect B. bigemina antibodies was a modification by Todorovic et al<sup>82</sup> of the original test described by Mahoney.<sup>43</sup> All serum samples found to be positive by these screening procedures were then titrated by the microprocedure technique as described by Hidalgo and Dimopoulos.<sup>25</sup> Antigen used in the complement fixation test for anaplasmosis was obtained from the United States Department of Agriculture.<sup>5</sup> Antigen used in the complement fixation test for babesiosis was obtained by the method described by Mahoney using a distilled water extract of the parasite suspension.<sup>41</sup> Antigen thus obtained was stored at -76° C until used in the complement fixation test.

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<sup>r</sup> Gelman Automatic Recording and Intergrating Scanner, No# 39372, Gelman Instrument Company, Ann Arbor, Mich.

<sup>5</sup> Animal Disease Eradication Branch, Agricultural Research Service, U.S.D.A.

## RESULTS

## EXPERIMENTAL PROCEDURES

Clinical Observations. Slight elevations of body temperatures were observed in the 4 groups of calves. An average maximum elevation of body temperature of 1.3°C. was observed in the calves infected with A. marginale only. The slight elevation of temperature occurred during the period when Anaplasma marginale bodies were first observed in the erythrocytes of the infected calves. The temperature of the calves returned to normal within 10 to 15 days and remained normal for the remainder of the experiment. No elevation of body temperature was observed in the calves infected with B. bigemina.

Body weight increases and average daily gains were calculated by finding the difference between initial weights on day 0 and the final weights on day 120 P.I. (Table 2).

The general physical condition of the calves of Groups I, III and IV declined as indicated by poor body condition, dull haircoats and inactivity. The calves of Group II remained active and in good physical condition throughout the experiment.

Hemoglobinuria was observed in calf #134 of Group III. The calf developed hemoglobinuria on day 7 following inoculation of blood containing B. bigemina, and died on day 9 P.I.

Gross Pathology. Calf # 134 of Group III, which was in the recovery phase of anaplasmosis, died on day 79, 9 days after being inoculated with blood containing B. bigemina. Gross lesions observed during necropsy of the calf included: hepatomegaly with yellow discoloration; splenomegaly and congestion; petechial hemorrhages in the renal capsule, pericardium, prefemoral and renal lymph nodes; distention of the urinary bladder with port wine

colored urine; distention of the gall bladder with thick yellow colored bile; the pericardium contained approximately 25 ml of blood stained fluid; the kidneys were dark red in color; the right ventricle of the heart was dilated and flacid; the mucus membranes of the mouth and the conjunctival membranes were pale; the lymph nodes were generally enlarged with prominent lymphoid follicles on the cut surface. The lungs remained inflated with air.

Two calves from each of Groups III and IV and a normal noninfected control calf were euthanatized and subjected to necropsy. Gross lesions observed during the dissection of the 4 infected calves included: from 500 to 1000 ml of straw colored fluid in the peritoneal cavity, 100 to 200 ml of fluid in the pleural cavity, and occasional fluid in the pericardial sac; moderate lymph node enlargement; splenomegaly with prominent splenic follicles; renal congestion; moderate hepatomegaly; serous atrophy of depot fat was occasionally observed. No differences in the extent or severity of gross lesions were observed between the 2 calves of Group III and the 2 calves of Group IV. An additional calf from Group III and 2 calves from Group IV died during the experiment. Gross and histopathological examinations indicated the 3 calves died from extraneous causes.

Microscopic Pathology. The spleen of calf #134, which died during the experiment, was severely congested and had focal areas of necrosis in the lymphoid follicles. Granules of hemosiderin were observed in reticulo-endothelial cells in the spleen, renal glomerular tufts, hepatic Kupffer cells and within alveolar septal macrophages in the lungs. The liver was moderately congested and numerous individual hepatocytes were undergoing degenerative and necrotic changes in the centralobular areas. Kupffer cell hypertrophy and erythrophagocytosis were prominent. Emphysema was observed

in most of the pulmonary lobules, and a moderate infiltration of lymphocytes and reticulo-endothelial cells were observed in the peribranchial areas. The basement membranes of the glomerular tufts were relatively thickened. Hemoglobin casts were observed in the lumens of several renal tubules. Necrosis of individual lymphocytes was observed in the lymphoid follicles of the lymph nodes. Numerous nucleated erythrocytes were observed in the sinuses of the lymph nodes, spleen and in the sinusoids of the liver.

Tissue sections from each of the 4 calves euthanatized for dissection were grouped together by organ and examined for microscopic abnormalities.

Liver. Slight to moderate centrilobular degeneration of the hepatocytes was observed, as evidenced by numerous hepatocytes having pyknotic and karyorhectic nuclei and eosinophilic cytoplasm. A slight to moderate infiltration of lymphocytes and reticulo-endothelial cells was observed in the periportal areas. The hepatic sinusoids contained an excessive number of mononuclear inflammatory cells, and immature nucleated erythrocytes. Kupffer cell hypertrophy was observed in most of the sections. Erythrophagocytosis and phagocytized hemosiderin granules were occasionally observed.

Kidney. An eosinophilic staining, proteinaceous material was observed in Bowman's capsules and in the lumens of the renal tubules. A slight infiltration of lymphocytes and plasma cells was occasionally observed in the interstitial tissues and was predominantly perivascular in distribution. Phagocytized hemosiderin granules were occasionally seen in the glomerular tufts and in the interstitium.

Spleen. A moderate to marked lymphoid hyperplasia of the malpighian corpuscles was commonly observed. Lymphoid exhaustion, as indicated by

the reduction in width or the absence of a zone of mature lymphocytes in the pericorpuscular areas, was evident in most sections. Hyperplasia of reticulo-endothelial cells in the red pulp was observed in some sections. Grannules of phagocytized hemosiderin were observed in the sinuses of all sections.

Lymph nodes. Histopathological examinations were conducted on tissue sections prepared from the suprathyngal, prescapular, prefemoral, sternal, bronchial, hepatic, renal and mesenteric lymph nodes. The microscopic abnormalities observed were similar irregardless of which lymph node was examined. Moderate hyperplasia of the lymphoid follicles was observed in all sections, with marked hyperplasia being observed in several sections. Hyperplasia of reticulo-endothelial cells was occasionally observed. Grannules of phagocytized hemosiderin were present in several sections.

Lung. Microscopic abnormalities were seldom observed and consisted of occasional perivascular and interstitial infiltration of lymphocytes in low numbers.

Cerebrum. Microscopic abnormalities were irregularly observed and consisted of occasional perivascular infiltration of low numbers of lymphocytes and plasma cells.

Adrenal and thyroid glands. No microscopic abnormalities were observed.

No differences in the extent or severity of histologic lesions were observed between the calves of Group III and Group IV.

Clinical pathological results are given in the following tables and figures.

TABLE 1.

The Separation of Babesia bigemina from Babesia argentina and Anaplasma marginale by the Rapid Passage of Infected Blood Through 5 Splenectomized Calves.

Splenectomized Calf No.	Incubation Period (Days)		
	<u>Babesia bigemina</u>	<u>Babesia argentina</u>	<u>Anaplasma marginale</u>
1	1-1/2	4	44
2	1-1/4	4	Neg.
3	1	15	Neg.
4	1-1/2	Neg.	Neg.
5	2-1/2	Neg.	Neg.

Neg. - No parasites seen in Giemsa Stained Blood Smears.

TABLE 2. Initial and Final Weights with Total Weight Gains and Average Daily Gains of the Four Groups of Experimental Calves.

Groups of Experimental Calves	Number of Calves per Group	Initial Weight Day 0 (kg)	Final Weight Day 120 (kg)	Total Gain (kg)	Average Daily Gain (kg)
I	3	135	182	47	0.39
II	3	137	240	103	0.86
III	8	137	183	46	0.38
IV	8	127	180	53	0.44

\*Weights are based on the average for the group.

\*\* Initial and final weights are based on the average of measurements made on 2 successive days at the beginning and termination of the experiment.

TABLE 3. Reticulocyte Counts of the 4 Groups of Experimental Calves.

Days	Groups of Experimental Calves			
	I %	II %	III %	IV %
-4	NS	NS	NS	NS
2	NS	NS	NS	NS
5	NS	NS	NS	NS
9	NS	NS	NS	NS
12	NS	NS	NS	NS
14	0.03	NS	NS	0.03
16	NS	NS	NS	NS
20	NS	NS	0.03	0.4
22	0.3	0.7	0.8	0.9
26	0.2	0.8	0.2	0.4
29	0.1	0.1	0.2	1.8
33	0.3	ND	ND	ND
36	0.5	1.2	2.9	3.3
40	ND	ND	ND	ND
43	0.3	0.3	0.2	0.3
47	0.3	0.3	0.2	0.3
50	0.4	0.8	0.7	0.4
54	ND	ND	ND	ND
61	0.2	0.2	0.2	0.1
64	0.2	0.1	0.2	0.2
68	0.2	0.3	0.5	0.4
71	0.3	0.2	0.2	0.4
75	0.6	0.6	0.8	0.3
82	0.3	0.5	0.3	0.4
85	0.8	0.4	0.4	0.4
89	0.3	0.3	0.5	0.3
92	0.3	0.1	0.1	0.3
96	0.2	0.1	0.1	0.3
99	0.1	0.1	0.1	0.4
103	0.1	0.1	0.1	0.3
110	0.1	0.1	0.1	0.2
117	0.1	0.1	0.1	0.2

\*NS -- Not seen

\*\*ND -- Not determined

TABLE 4--Serum Sorbitol Dehydrogenase Concentrations. Group means are Presented in Sigma Units/ml of Serum.

Groups of Experimental Calves	DAY									
	-12	-10	+12	26	40	54	68	82	96	110
I	307	665	480	339	568	500	ND	ND	634	1154
II	493	558	613	593	388	446	ND	ND	261	586
III	467	503	426	516	300	391	ND	ND	259	826
IV	423	415	411	666	296	299	ND	ND	333	1353

\*ND -- Not determined

TABLE 5--Myeloid:Erythroid Ratio as Determined by the Examination of Bone Marrow Biopsies Collected on Days 0, 50, 90 and 120 of the Experiment.

Groups of Experimental Calves	Calf No.	M:E Ratio			
		Day 0	Day 50	Day 90	Day 120
I	122	0.59	0.23	0.40	0.57
	123	0.56	0.17	0.20	0.23
II	127	0.53	ND	0.36	0.24
	128	0.60	ND	ND	0.64
III.	133	0.78	0.25	0.15	0.18
	144	0.64	0.34	0.36	0.34
IV	147	0.49	0.30	0.14	0.13
	149	0.55	0.28	0.22	ND

\*ND -- Not determined

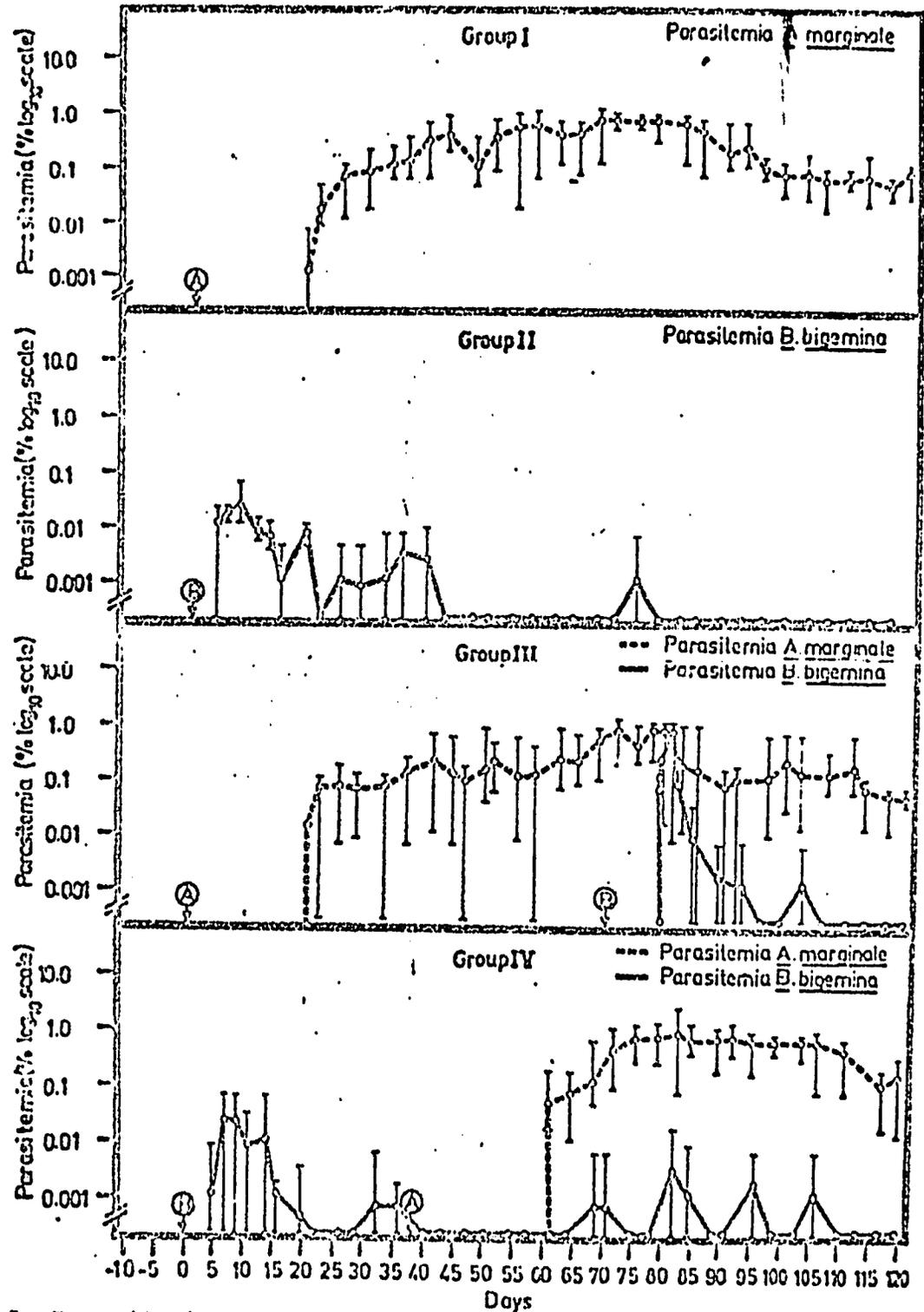


Fig. 1. Parasitemia. Group means are presented and vertical lines represent range. Encircled letters indicate time of intravenous inoculation with Anaplasma marginale or with Babesia bigemina.

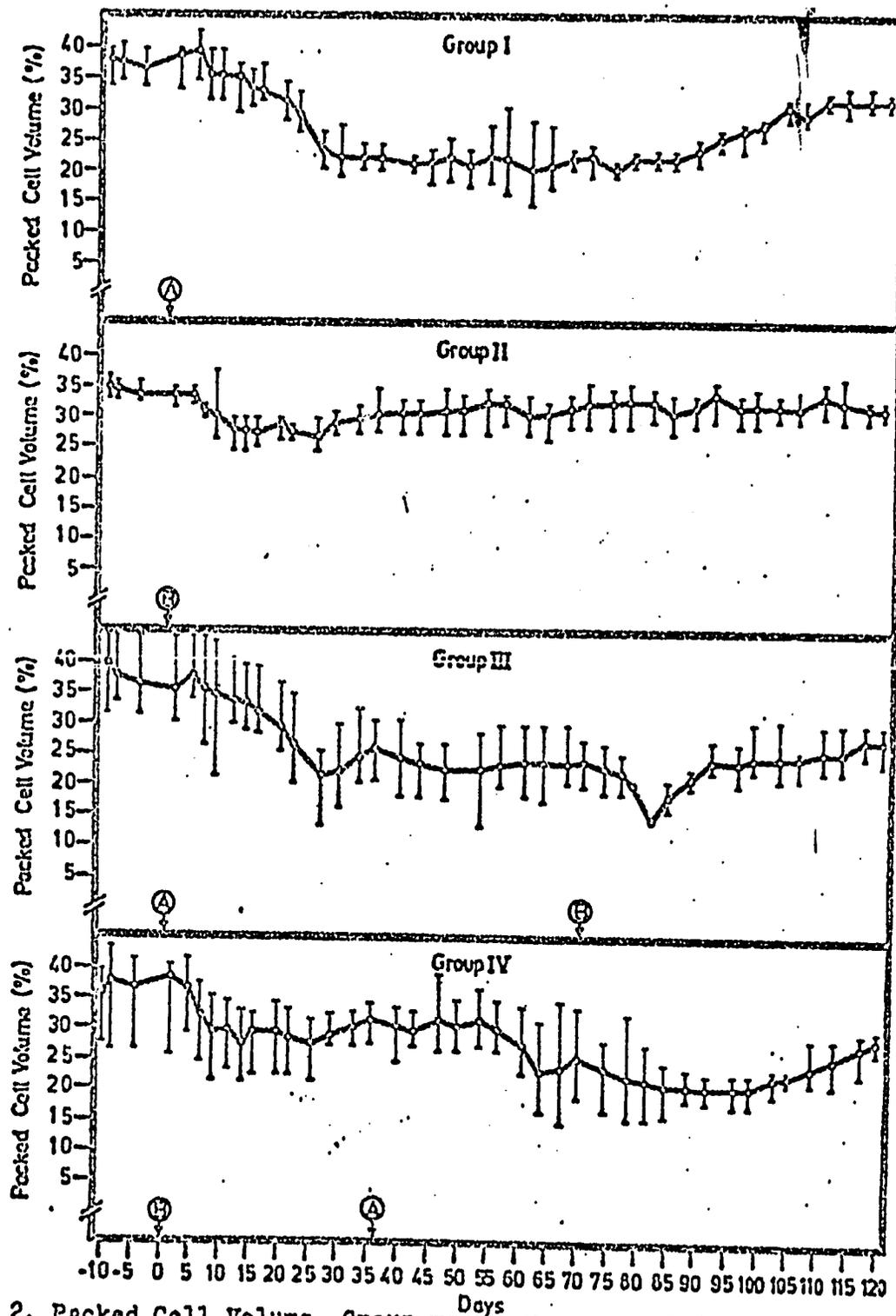


Fig. 2. Packed Cell Volume. Group means are presented and vertical lines represent range. Encircled letters indicate time of intravenous inoculation with Anaplasma marginale or with Babesia bigemina.

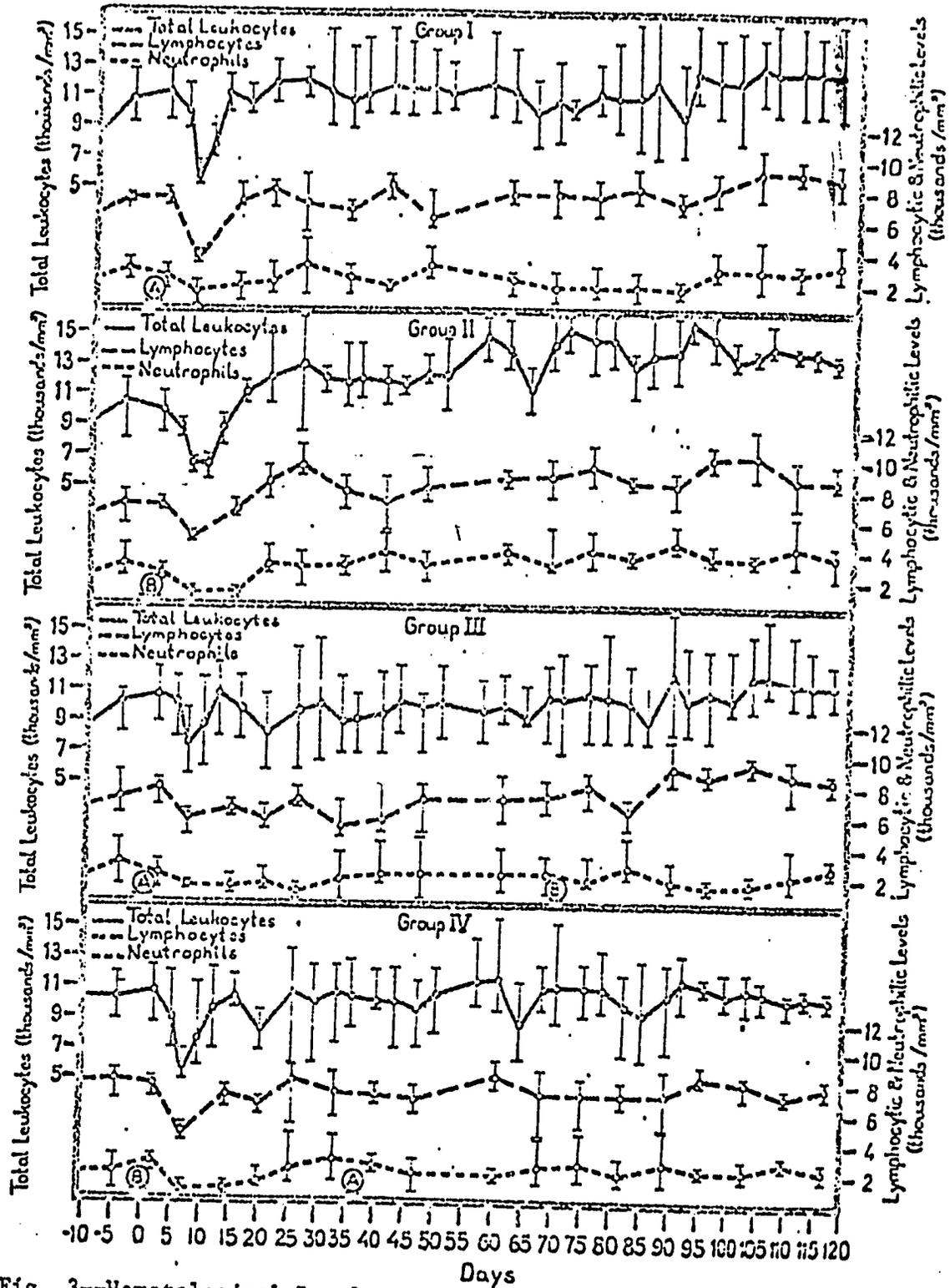


Fig. 3--Hematological Results. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale or with Babesia bigemina.

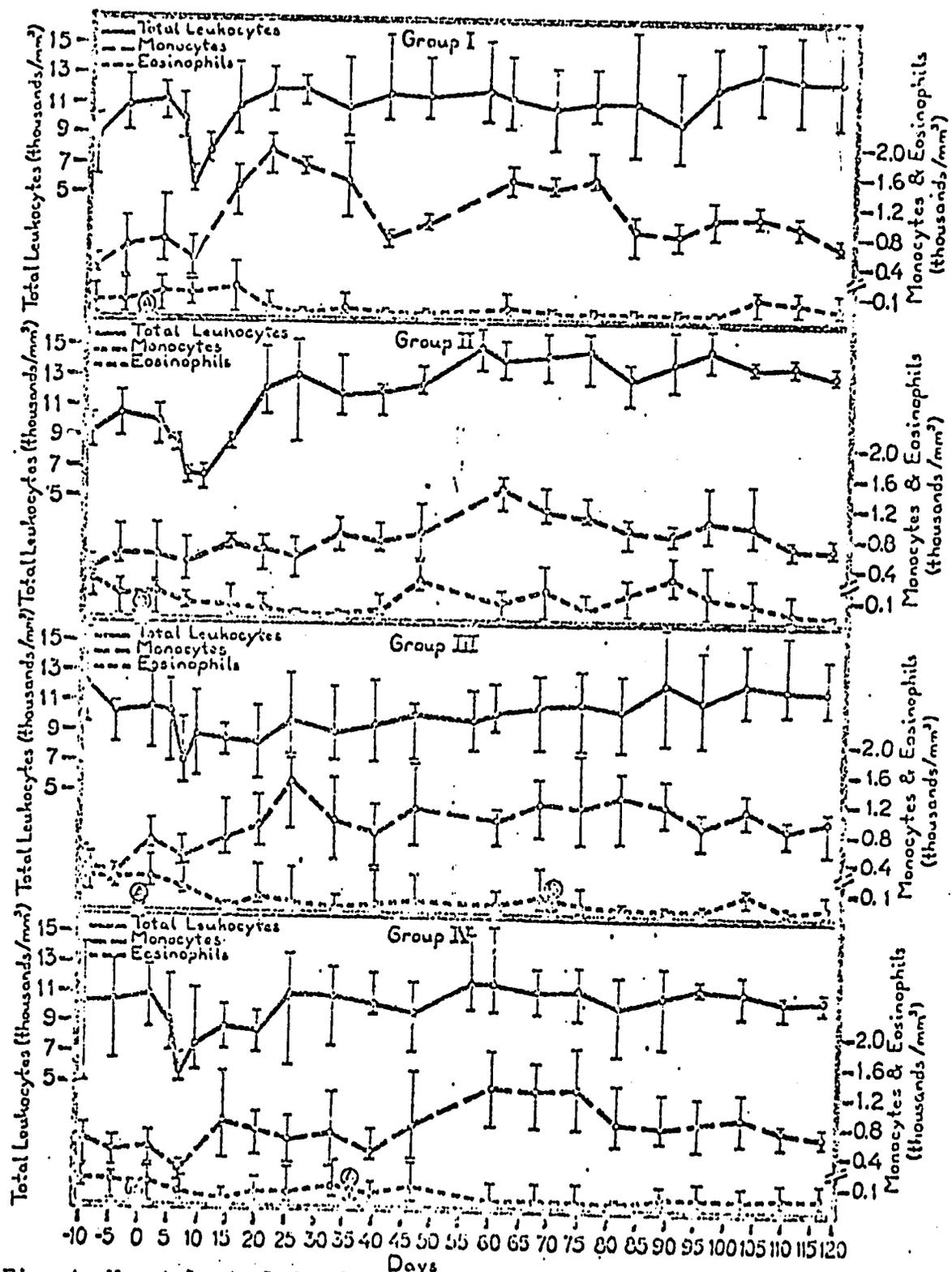


Fig. 4. Hematological Results. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale or with Babesia bicemina.

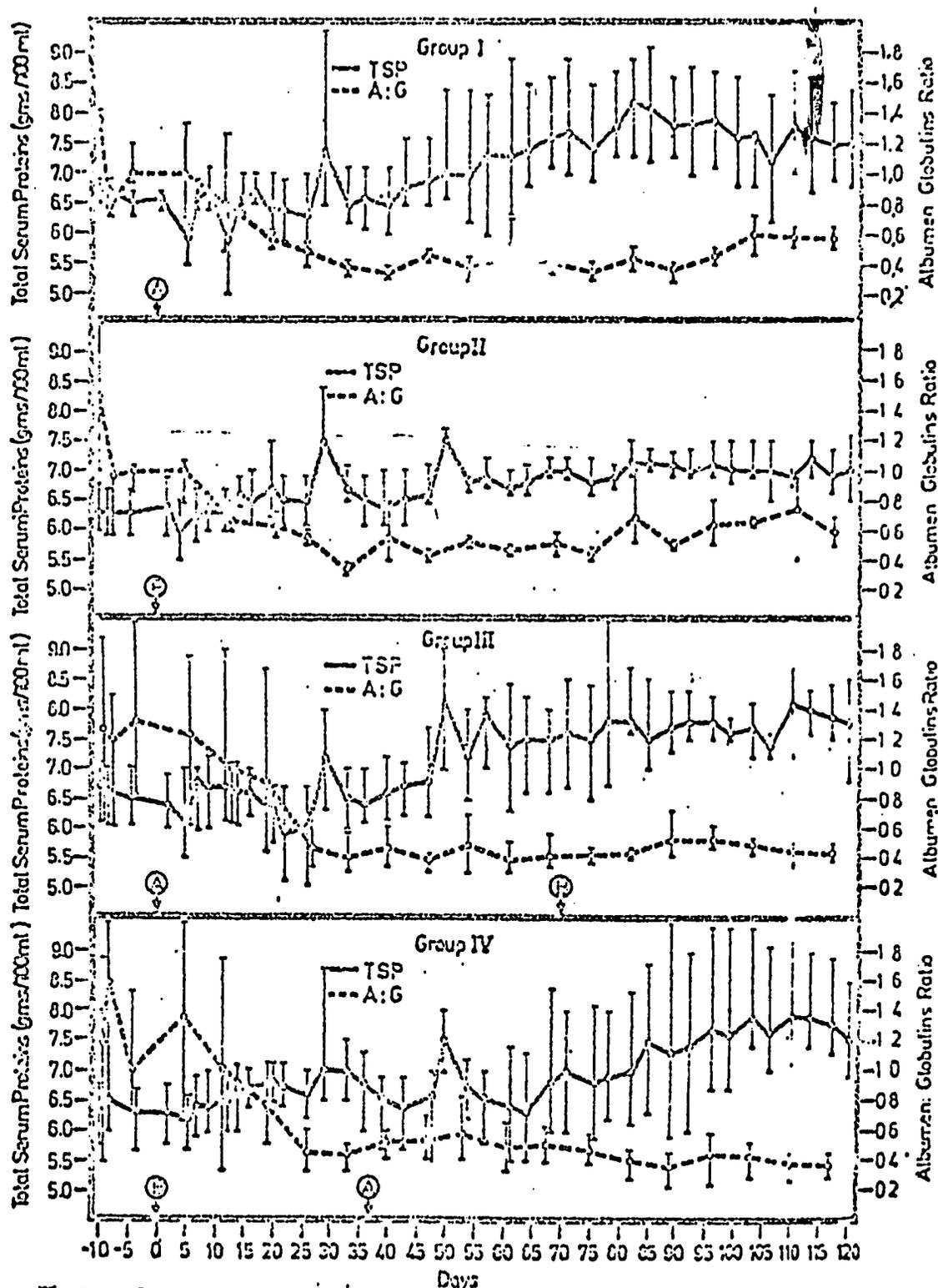


Fig. 5. Total Serum Proteins and Albumin:Globulin Ratio. Group means are presented and vertical lines represent range. Encircled letters indicate time of *Sus scrofa* inoculation with Anaplasma marginale or with Babesia bigemina.

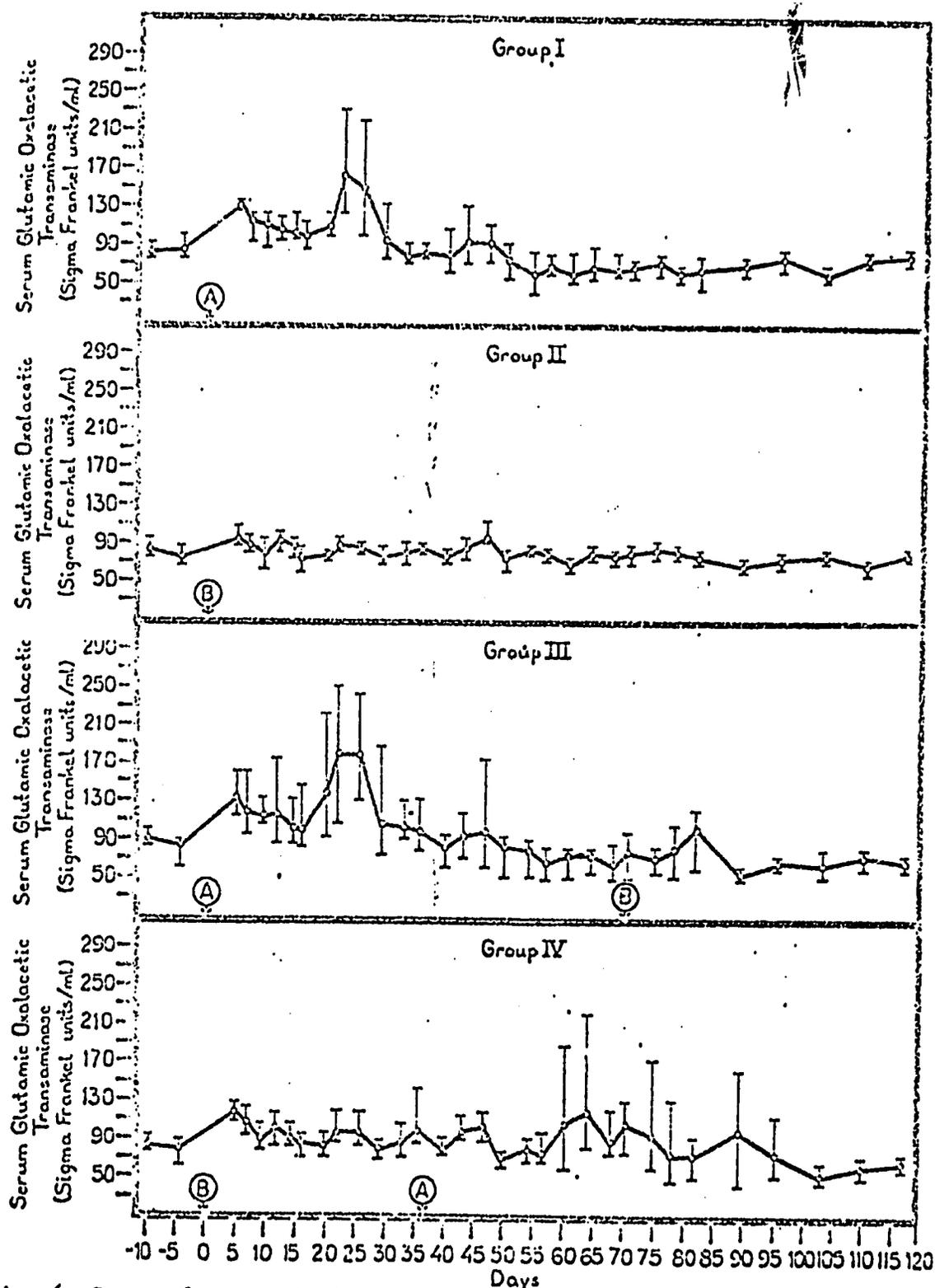


Fig. 6. Serum Glutamic Oxalacetic Transaminase Levels. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale or with Babesia bigemina.

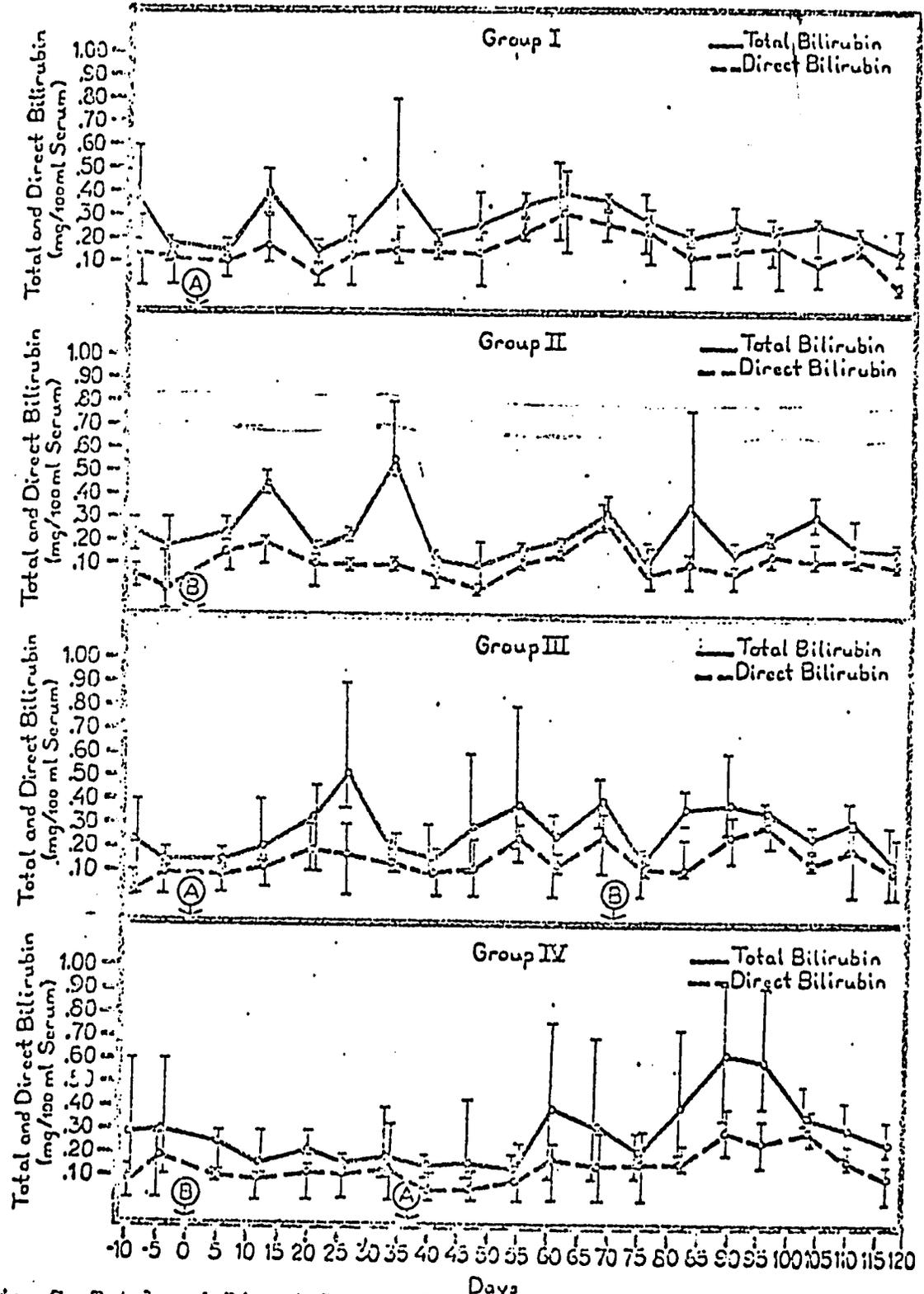


Fig. 7. Total and Direct Serum Bilirubin Concentrations. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with *Anaplasma marginale* or with *Babesia birewina*.

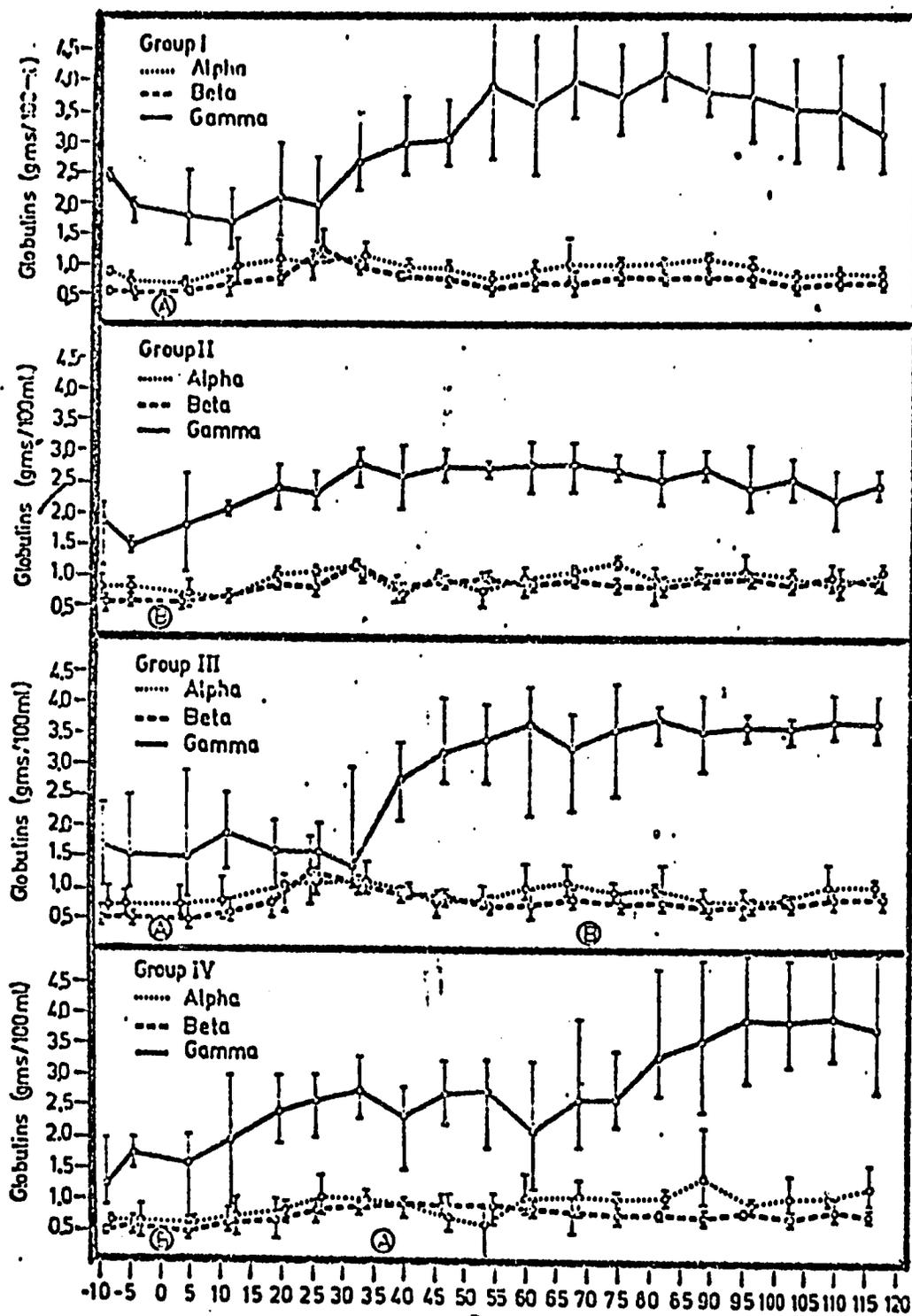


Fig. 3. Results of Serum Protein Electrophoresis. Alpha, Beta and Gamma Globulin fractions are presented. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with *Anaplasma marginale* or with *Babesia bigemina*.

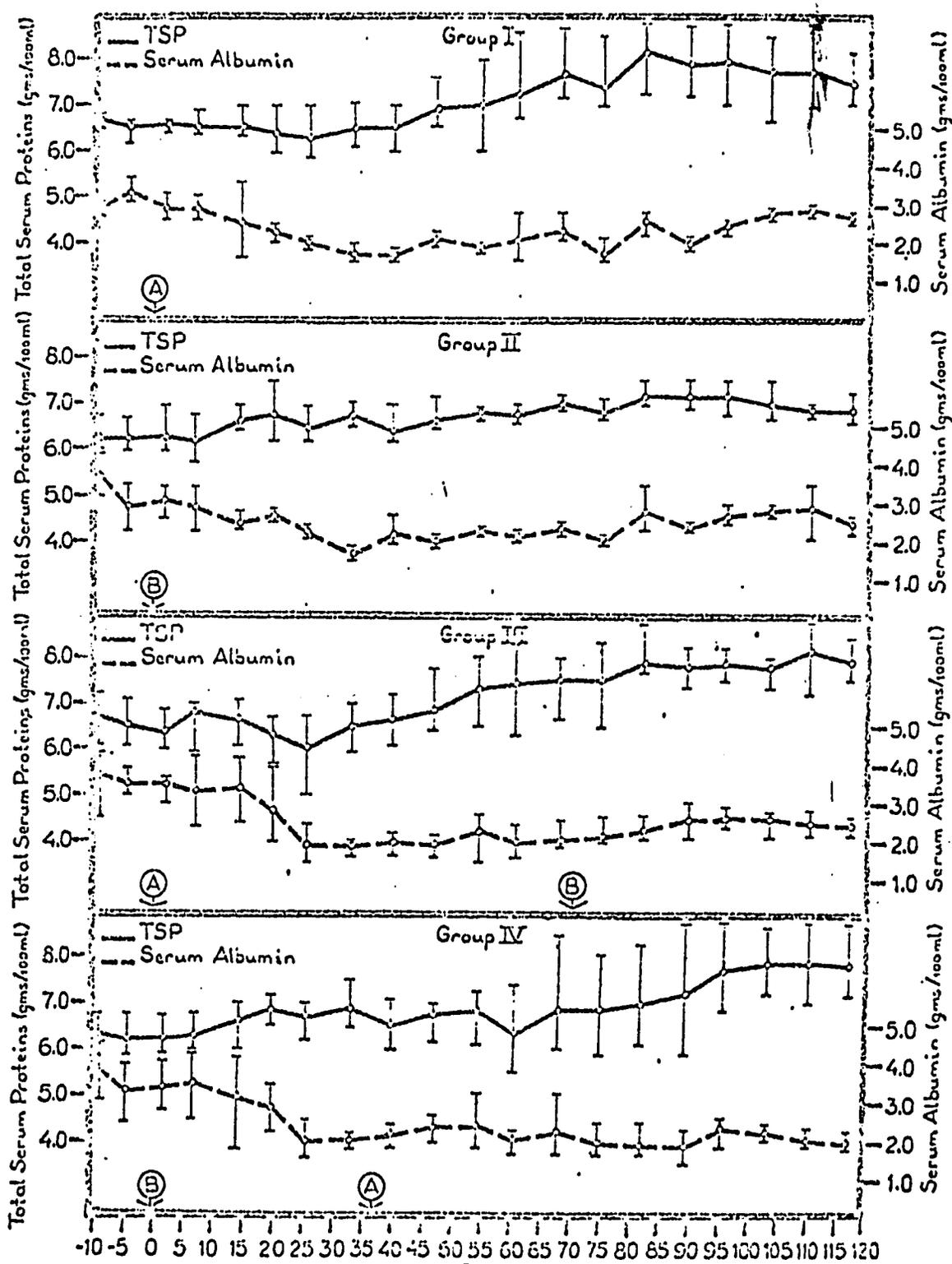


Fig. 9. Total Serum Proteins and Serum Albumin Concentrations. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with *Anaplasma marginale* or with *Babesia bireminia*.

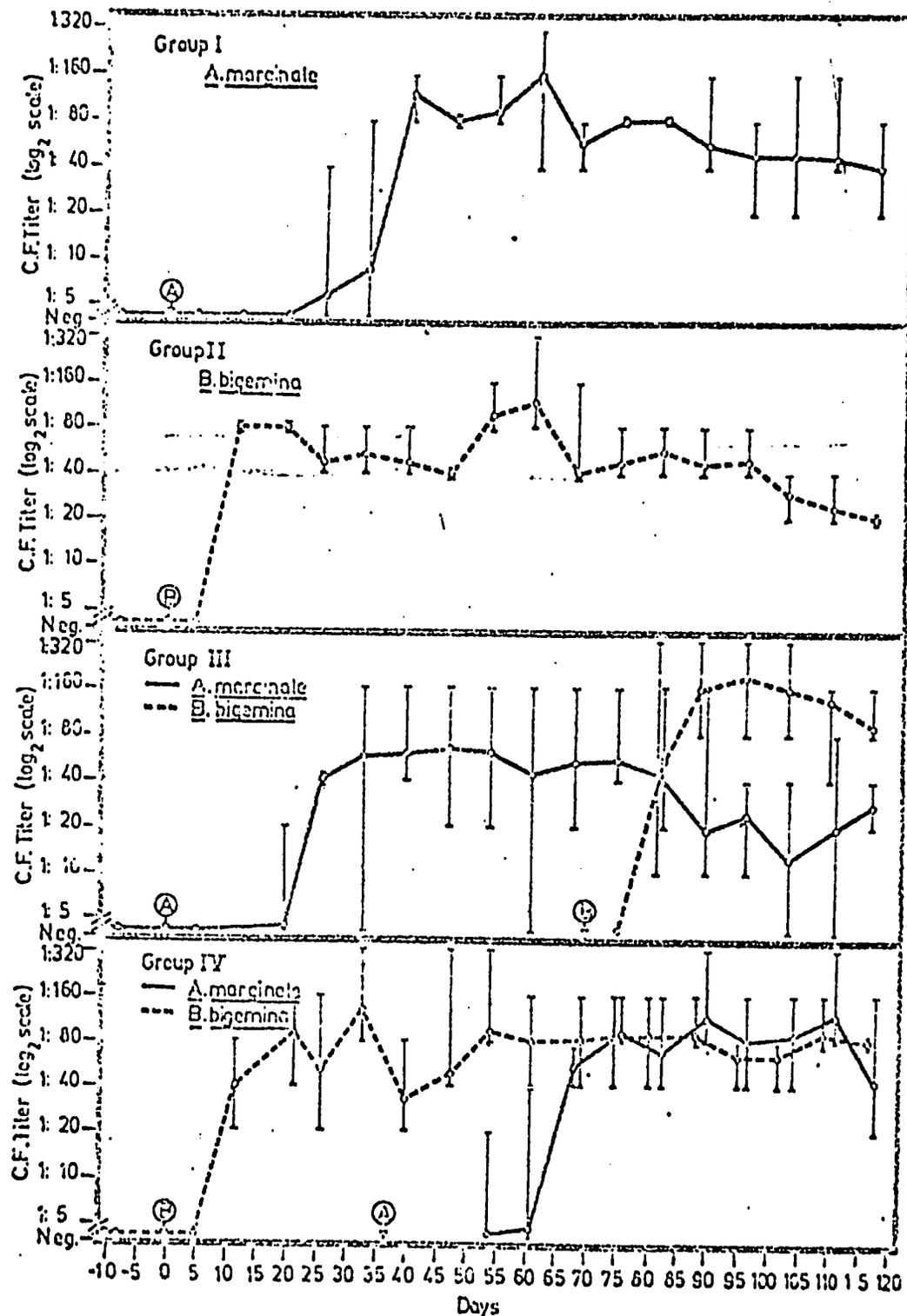


Fig. 10. Complement Fixing Antibody Titers. Group means are presented and vertical lines represent range. Encircled letters indicate time of *Subcutaneous* inoculation with *Anaplasma marginale* or with *Babesia bigemina*.

## SUMMARY

Isolates of A. marginale and of B. bigemina were obtained by the natural tick transmission of the organisms to susceptible calves under field conditions. Babesia bigemina was separated from B. argentina and A. marginale by the rapid serial passage of infected blood through 5 splenectomized calves. Anaplasma marginale was therapeutically separated from B. bigemina and B. argentina utilizing a babesiacidal drug at a sterilizing dosage. Following the addition of glycerol as a cryoprotectant the isolates of A. marginale and B. bigemina were frozen and stored at -79 C.

Twenty two, 7 month old, male, Holstein-Friesian calves were divided into 4 experimental groups. Groups I and II consisted of 3 calves each and served as control groups for anaplasmosis and babesiosis respectively. Groups III and IV consisted of 8 calves each and served to study concurrent babesiosis in calves recovering from anaplasmosis, and concurrent anaplasmosis in calves recovering from babesiosis respectively. The calves were infected with A. marginale and B. bigemina by the subcutaneous inoculation of 5 ml of infected blood containing approximately  $9.5 \times 10^8$  and  $10.5 \times 10^8$  organisms respectively.

Clinical manifestations of disease were mild and consisted of slight fever, poor carcass condition, poor weight gain, and inactivity. Hemoglobinuria was observed in one calf which died 9 days after being infected with B. bigemina. Fever was observed only in the calves which were infected with A. marginale. Poor carcass condition, poor weight gain, and inactivity were observed in the control calves of Group I and in the concurrently infected calves of Groups III and IV, and were attributed predominantly to infection with A. marginale.

Associated with the appearance of parasitized erythrocytes were decreases in PCV, hemoglobin concentration, albumin:globulin ratio, and serum albumin, and slight increases in SGOT, serum bilirubin concentration, and in the concentration of alpha and beta globulins. Changes associated with an increase in the number of parasitized erythrocytes included the appearance of reticulocytes in the peripheral blood; a decrease in the myeloid:erythroid ratio; and increases in the concentrations of total serum proteins, gamma globulins, and in serum sorbitol dehydrogenase.

Slight fluctuations were observed in the total leukocyte counts. A prolonged monocytosis was observed in each of the 4 groups of calves, while the number of neutrophils, basophils, eosinophils and lymphocytes remained relatively stable.

Decreases in PCV and in hemoglobin concentration were more severe and prolonged in the concurrently infected calves of Groups III and IV, and in the anaplasmosis control calves of Group I. The slight decrease in PCV observed in the babesiosis control calves of Group II suggested the severe anemic condition observed in the concurrently infected calves was due predominantly to infection with A. marginale.

Incubation periods were slightly prolonged in the concurrently infected calves. The loss of viability and/or infectivity of the Anaplasma and Babesia organisms, following prolonged storage at -79 C probably caused the slightly prolonged incubation periods observed.

The number of parasitized erythrocytes was observed to be higher in the concurrently infected calves than in the control calves. The increased number of infected red cells may have been attributable to a moderate decrease in the ability of the reticulo-endothelial system to respond to infection in the concurrently infected calves allowing the secondary organism to reach higher numbers in the peripheral blood.

A concurrently infected calf which died from acute babesiosis, and 2 concurrently infected calves from each of Groups III and IV were subjected to necropsy. The lesions observed in the 4 euthanatized

calves were moderate in extent and severity and it was presumed the calves would have survived the natural course of the concurrent infections.

Gross lesions observed included the presence of a moderate amount of yellow fluid in the peritoneal and pleural cavities; moderate lymph node enlargement; moderate splenomegaly and hepatomegaly; renal congestion; and occasional serous atrophy of depot fat. Gross lesions observed in the calf which died of acute babesiosis involved essentially the same organs as in the euthanatized calves, but were more severe and extensive and included hemoglobinuria.

Slight to moderate centrilobular degeneration and hepatocellular necrosis was observed and associated with hypoxia resulting from the anemic condition of the calves. Kupffer cell hypertrophy and lymphoid hyperplasia of the splenic malpighian corpuscles and lymphoid follicles were observed and attributed to the reaction of the reticulo-endothelial system to infection. Granules of hemosiderin were observed tissue sections from the spleen, liver, kidney and lymph nodes and were attributed to the removal of eryth release of the breakdown products of hemoglobin.

Microscopic abnormalities observed in tissue sections from the lung, cerebrum, cerebellum, adrenal and thyroid glands were slight and inconsistent, and were considered not to have resulted from infection with A. marginale or with B. bigemina.

Complement fixing antibodies for anaplasmosis were first observed on days 17 to 26 PI and corresponded to the increase in alpha and beta globulin. The increase in CF antibody titer corresponded to the increase in gamma globulin and persisted until the concentration of gamma globulin began to recede during the terminal days of the experiment.

CF antibodies for babesiosis were first observed on day 12 PI and corresponded to a general increase in the concentration of the alpha, beta and gamma globulins. A increase in CF antibody titer for babesiosis corresponded to an increase in the concentration of gamma globulin. A decrease in antibody titer was observed as the concentration of gamma globulin decreased near the end of the experiment.

IMMUNOSEROLOGIC CHARACTERIZATION OF BABESIA ARGENTINA AND BABESIA BIGEMINA  
ANTIGENS BY COMPLEMENT FIXATION; GEL PRECIPITATION AND IMMUNOELECTROPHORESIS

INTRODUCTION

Bovine babesiosis in Colombia causes economic losses in cattle and its diagnostic of the chronic stage of the disease makes it necessary to realize epidemiologic studies in order to control the disease. The disease is produced by cross infections of B. argentina, B. bigemina and B. major (10), which sometimes are associated with anaplasmosis and trypanosomiasis infections (3, 7, 11). It is often difficult to obtain a positive blood smear in the chronic stages of the disease, therefore, it is necessary to obtain specific antigens for the serologic tests such as complement fixation (FC) and gel precipitation (GP) to detect different types of antibodies against the parasite (4, 5, 8).

This work was realized in the Laboratorio de Investigaciones Médicas Veterinarias with the cooperation of the Texas A & M University Mission in Colombia and had the following objectives: 1) to obtain specific antigens for the serologic CF and GP diagnosis of B. bigemina and B. argentina, (2) to compare the antigenicity by serology and cross immunity in calves infected with B. bigemina and B. argentina.

MATERIALS AND METHODS

Thirty five calves, Holstein, 3 - 4 - months of age were bought from farms in the Sabana de Bogotá which is apparently Boophilus microplus free. These calves were divided in 6 groups for serologic tests, to isolate strains of Babesia, to obtain antigens and for the cross immunity tests. Nine adult

rabbits were utilized for production of antisera for the GP and immunoelectrophoresis (IE) tests.

The strains of B. bigemina (1) and B. argentina were isolated from splenectomized calves infected with B. microplus in Turipaná and were further purified from other infecting organisms by rapid serial passage in splenectomized calves.

B. bigemina and B. argentina antigens for CF were separated from parasitized erythrocytes by a modified method of Mahoney (6). One part of the antigenic fraction was lyophilized and other was frozen at  $-79^{\circ}\text{C}$ .

Soluble antigens were obtained passing the serum of animals with an acute infection of B. bigemina or B. argentina through columns of DEAE-cellulose and Sephadex G - 200. Fibrinogen was previously removed from the plasma by adding slowly with continuous mixing a 1 m calcium chloride solution. Microtechnique methods (3, 9) were utilized for the CF tests with sera of carrier animals of B. argentina and/or B. bigemina.

Soluble serum antigens were utilized in the GP tests of B. argentina and B. bigemina obtained from chromatography and their respective antisera were absorbed. The same antigens and antisera were utilized in IE during 60 minutes at 300V and 10 ma utilizing a slide box of 6 slides.

For cross studies, immunity 20 calves were divided into 2 groups of 10 each. Inoculations were made days 0 and 28 with the inoculation of infected parasitized erythrocytes of  $1 \times 10^{10}$ ,  $1 \times 10^7$  and  $1 \times 10^4$ . All calves were challenged on day 176 with  $1 \times 10^{10}$  infected erythrocytes. Parameters of CF titers, parasitemia, hematocrit, temperature and weight were also determined.

## RESULTS

During the 14 days that the splenectomized calf was kept in the pasture adjacent to the farm, larvae were shown in the 4th day, nymphs in the 11th day and in the 14th day the calf showed a rectal temperature of 41.4°C and 1.2% of B. argentina and cerebral babesiosis were observed.

The crude antigen of B. argentina was more antigenic than the four fractions obtained with distilled water. The fixation of the second fraction was more dense and none were anticomplementary. Of the 8 fractions obtained of B. bigemina, the first two were anticomplementary and the third showed a strongest antigenic reaction. With the columns of the anionic interchange of DEAE - cellulose, the separation of three protein peaks was found in the serum of calves with B. argentina or B. bigemina. The soluble antigen was found in the second protein peak. From the second protein peak separated by DEAE - cellulose, the pure antigen was obtained with sephadex G - 200.

Complement fixation showed antibodies of B. argentina or B. bigemina with aspect of 2 + beginning from the sixth day post-infection and from the 8th day, the titers were found to be between 1:10 - 1:20. The highest titers were detected in the carriers.

The antigens exhibited the strongest antigenicity when reacted homologously, however the antigens reacted slightly in a low percentage when were tested with heterologous sera.

The soluble antigen of B. argentina with its corresponding antisera showed the strongest precipitation and two bands of precipitation.

The B. bigemina soluble antigen reacted slightly, and only two precipitation bands were observed on the agar. Both showed slight precipitation of antibodies in the sera of animals recovered from babesiosis. In immunoelectrophoresis, both antigens had a slight mobility toward the negative pole and reacted with well defined archs.

In the cross immunity studies, B. argentina caused the strongest clinical reaction.

Please see attached tables and graphs for results.

#### DISCUSSION

Two types of antigenic characteristics were found: (1) species specific or distinct antigenic and characteristics for each species of organism (2) common genus or similar antigenic characteristics for both organisms. In addition, two groups of calves were infected with Babesia bigemina and Babesia argentina respectively, and 176 days after infection, they were challenged with the same and alternate parasites. It was found that Babesia bigemina and Babesia argentina parasites gave partial cross-protective immunity against one another., and B. argentina gave more protective immunity against B. bigemina than the contrary, therefore vaccines for protection against babesiosis should include B. argentina rather than B. bigemina.

## FIJACION DEL COMPLEMENTO CON LOS ANTIGENOS

B.argentina Y B.bigemina

<u>196 SUEROS <u>B.arg.</u> VS AG-<u>B.arg.</u></u>		<u>200 SUEROS <u>B.big.</u> VS AG-<u>B.big.</u></u>	
93	-	106	-
3	2+	1	ac
10	3+	1	1:10
1	ac	1	1:20
1	1:5	5	1:40
6	1:20	33	1:80
26	1:40	31	1:160
23	1:80	13	1:320
15	1:160	9	1:640
10	1:320		
7	1:640		
<u>200 SUEROS <u>B.big.</u> VS AG-<u>B.arg.</u></u>		<u>196 SUEROS <u>B.arg.</u> VS AG-<u>B.big.</u></u>	
182	-	184	-
6	1+	2	1+
4	2+	5	2+
2	3+	2	3+
1	ac	1	ac
	1:5	2	1:5
2	1:10		

LISIS DE ERITROCITOS PARASITADOS DE B. bigemina Y B. argentina CON NaCl

CONCENT. NaCl (%)	SANGRE 23% <u>B. arg.</u>		SANGRE 23% <u>B. big.</u>	
	% ERITR. NO LIS.	%   NO LIS.	% ERITR. NO LIS.	%  NO LIS.
53	60	31	50	21.5
47	30	66	20	37
42	20	83	0	0

(MAHONEY, 1.967)

SANGRE NORMAL

38 LISIS 100%

SANGRE 7.5% B. arg.

35  
 % ERITR. %   
 NO LIS. NO LIS.  
 6 +90

CARACTERISTICAS DE LA INFECCION POR Babesia bigemina Y POR Babesia argentina  
 OBTENIDA CON  $1 \times 10^{10}$ ,  $1 \times 10^7$  Y  $1 \times 10^4$  GLOBULOS ROJOS PARASITADOS

I N O C U L O	PARASITADOS INOCULADOS		PERIODO PREPATENENTE (DIAS)		MAXIMA PARASITEMIA (%)		TEMPERATURA MAXIMA (°C)		HEMATOCRITO MINIMO (%)		T I T U L O M A X I M O F.C.	
	<u>Bb</u>	<u>Ba</u>	<u>Bb</u>	<u>Ba</u>	<u>Bb</u>	<u>Ba</u>	<u>Bb</u>	<u>Ba</u>	<u>Bb</u>	<u>Ba</u>	<u>Bb</u>	<u>Ba</u>
$1 \times 10^{10}$	3/3	2/2	2.0	3.0	3.2	0.005	41.5	41.7	18	11	1:320	1:160
$1 \times 10^7$	3/3	3/3	5.7	3.0	0.03	0.003	40.5	41.6	27	17	1:160	1:640
$1 \times 10^4$	0/4	2/2	-	4.0	-	0.001	39.7	41.4	27	18.5	Neg.	1:640
$1 \times 10^{10}$ (56°C X 35')		2/3		4.5		0.004		41.1		18.5		1:320

\* UNICAMENTE EN Babesia argentina.

PASAJES RAPIDOS DE BABESIA bigemina Y BABESIA argentina EN TERNEROS ESPLENECTOMIZADOS

CON  $1 \times 10^6$  GLOBULOS ROJOS PARASITADOS

PASAJE No.	I N O C U L O		PERIODO PREPATENIE		P A R A S I T E M I A	
	<u>Bb</u>	<u>Ba</u>	<u>Bb</u>	<u>Ba</u>	<u>Bb</u>	<u>Ba</u>
1	180 ml.I.V.	100 ml.I.V.	36 h.	7 días	1.5	0.001
2	12 ml.I.V.	50 ml.I.V.	30 h.	4 días	0.5	0.01
3	**12 ml.I.V.	*15 ml.I.V.	24 h.	4 días	0.1	0.3
4	12 ml.I.V.	13 ml.I.V.	37½ h.	4 días	0.1	0.2
5	* 12 ml.I.V.	* 10 ml.I.V.	57½ h.	4 días	0.01	0.03

\* GANASEG

\*\* OXITETRACICLINA

Figura 19. Promedio del Título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso diario de 2 terneros inoculados ( I ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina y 4 semanas más tarde con una descarga (D) (Ba) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina. Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.

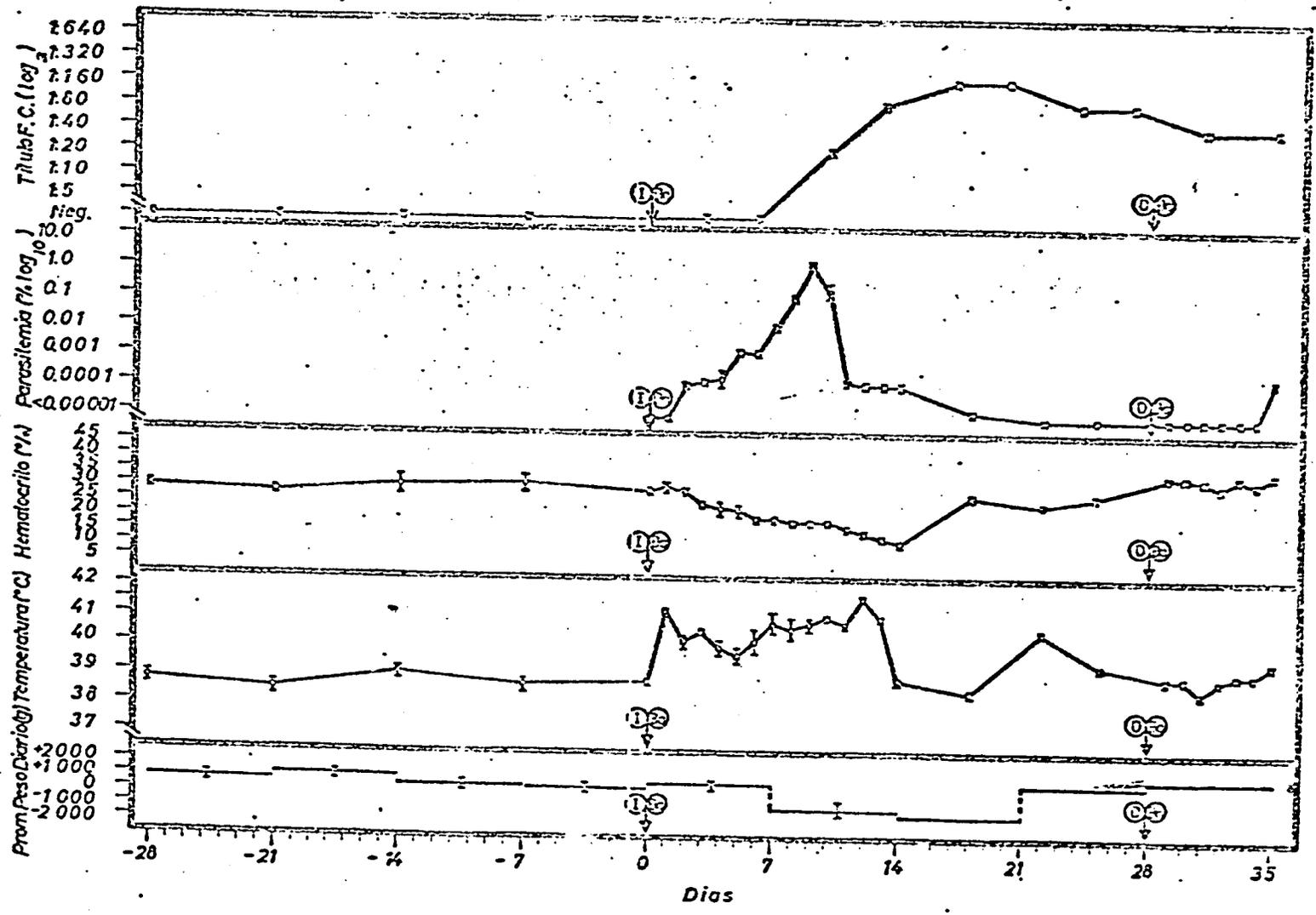


Figura 20. Promedio del título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso diario de 3 terneros inocularados ( I ) ( Ba ) con  $1 \times 10^7$  eritrocitos parasitados por B. argentina y 4 semanas más tarde con una descarga ( D ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina. Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.

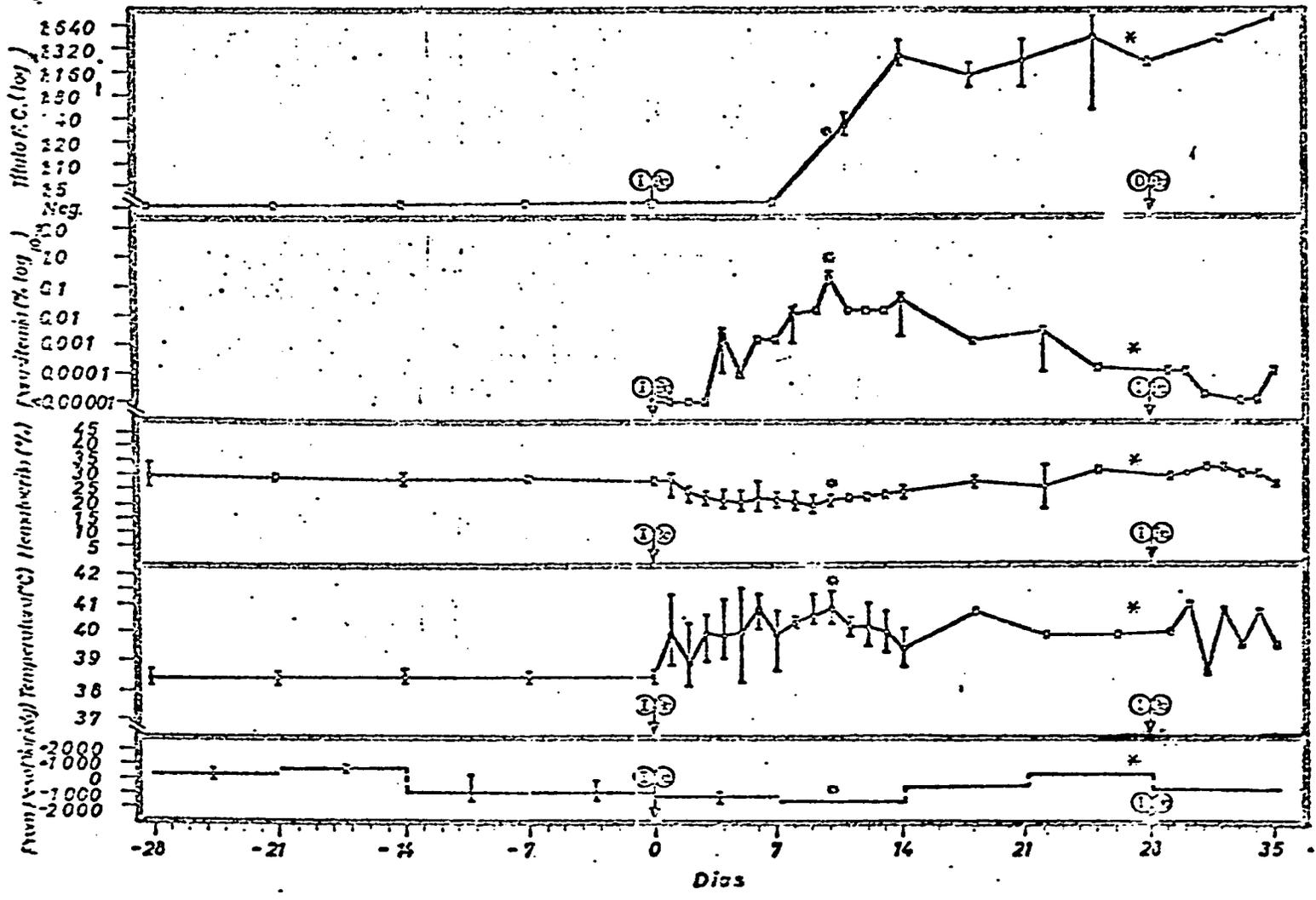


Figura 21. Promedio del título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso diario de 2 terneros inoculados ( I ) ( Ba ) con  $1 \times 10^4$  eritrocitos parasitados por B. argentina y 4 semanas más tarde con una descarga ( D ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina. Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.

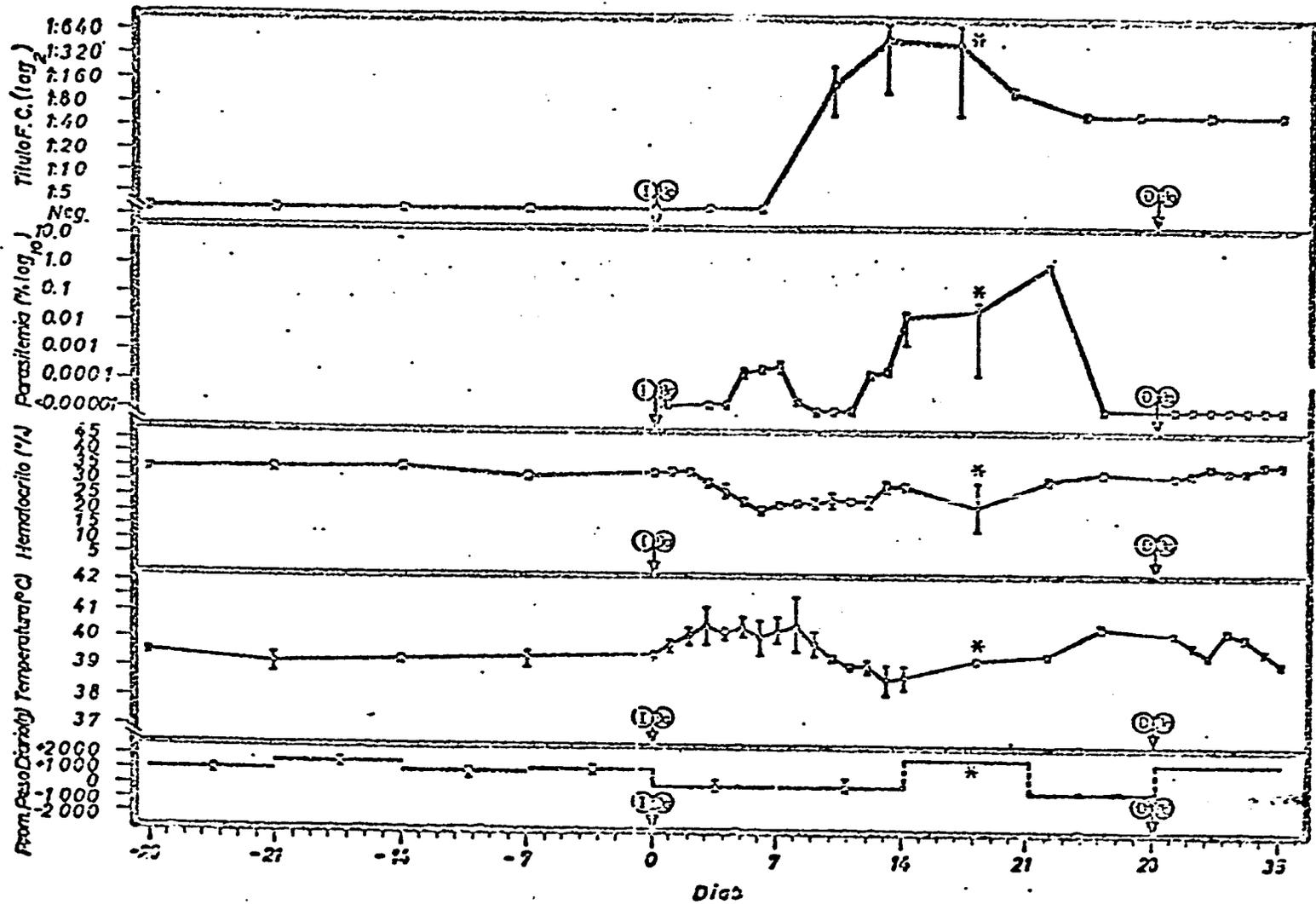


Figura 22. Promedio del título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso diario de 3 terneros inoculados ( I ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina e inactivados a  $56^{\circ}\text{C}$  por 35 minutos y 4 semanas más tarde con una descarga ( D ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina. Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.

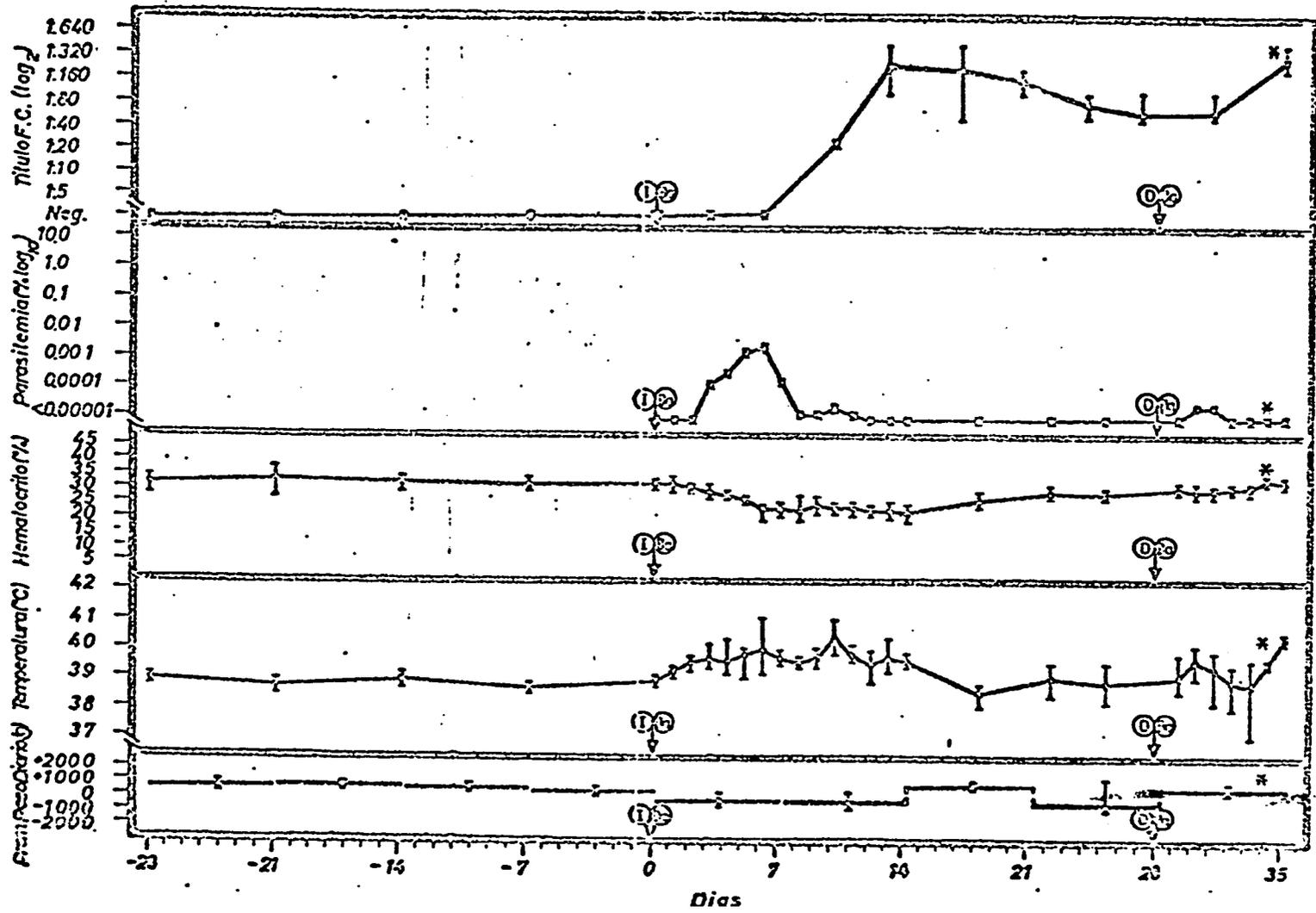


Figura 23. Promedio del título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso diario de 2 terneros inoculados ( I ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina, 4 semanas más tarde con una descarga ( D ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina y 4 meses después una segunda descarga ( D ) ( Bb ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. bigemina. Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.

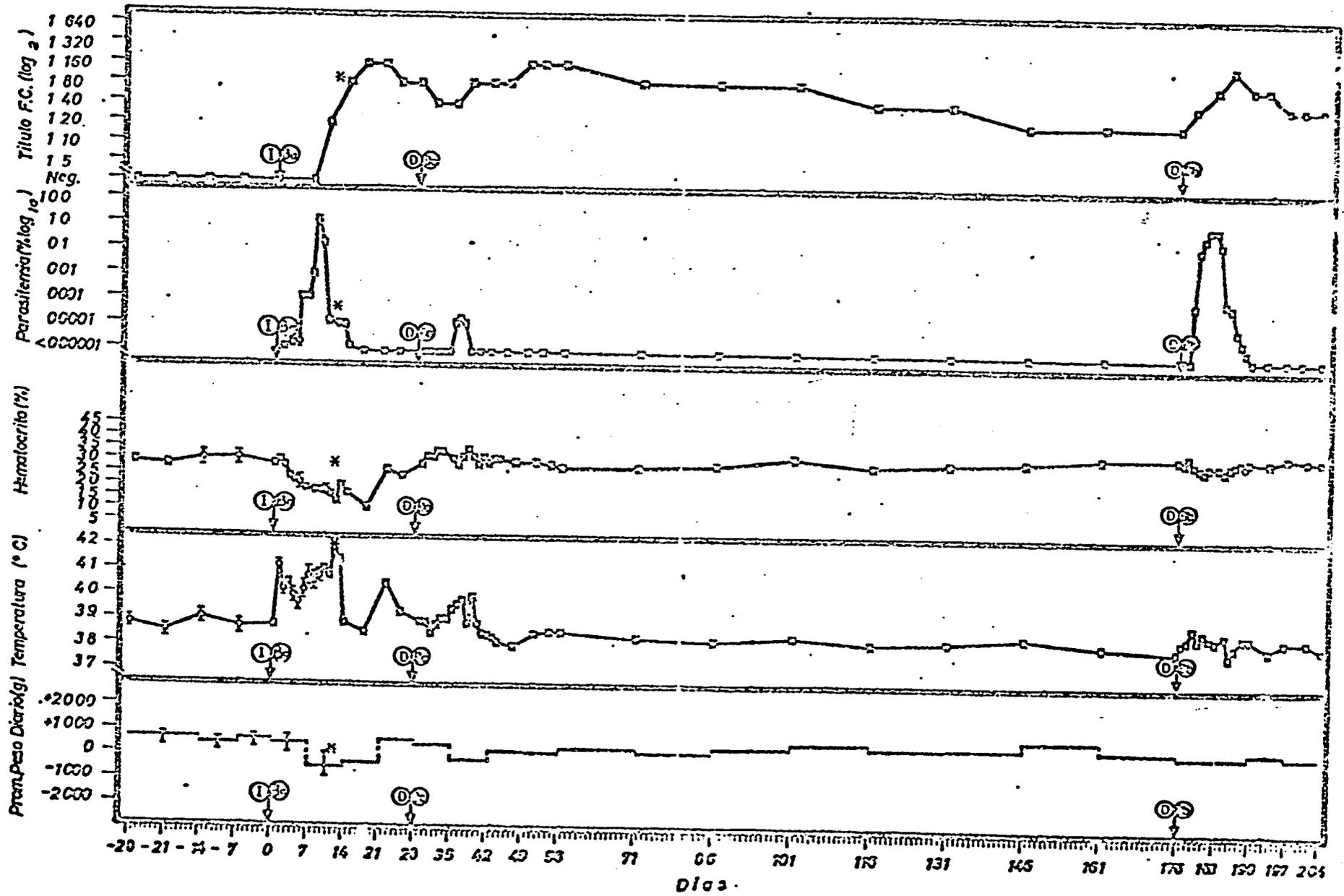


Figura 24. Promedio del título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso diario de 2 terneros inoculados ( I ) ( Bb ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. bigemina, 4 semanas más tarde con una descarga ( D ) ( Bb ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. ar  
gentina. Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.

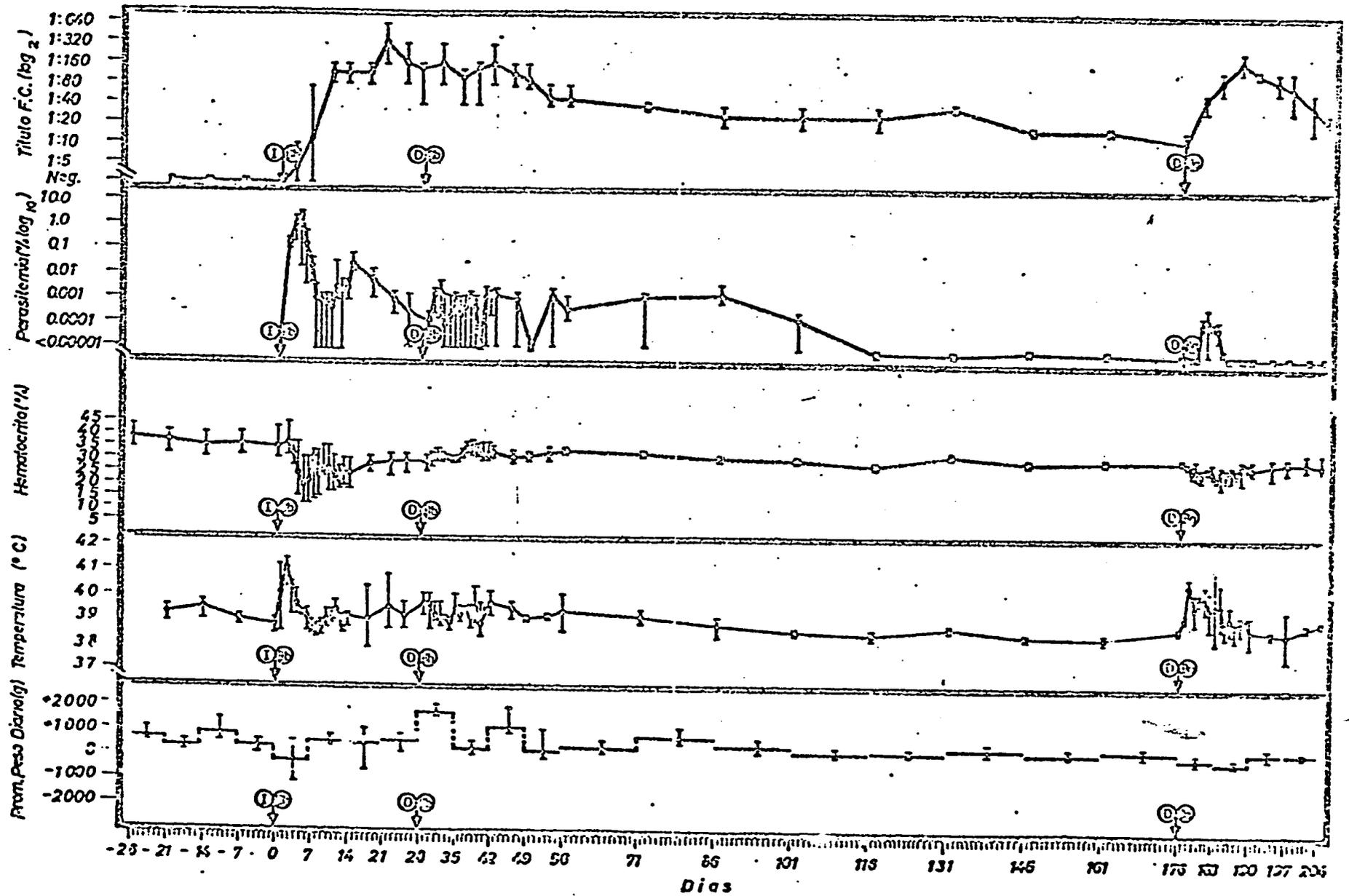


Figura 25. Promedio del título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso diario de 2 terneros inoculados ( I ) ( Ba ) con  $1 \times 10^4$  eritrocitos parasitados por B. argentina, 4 semanas más tarde con una descarga ( D ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina y 4 meses después una segunda descarga ( D ) ( Bb ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. bigemina. Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.

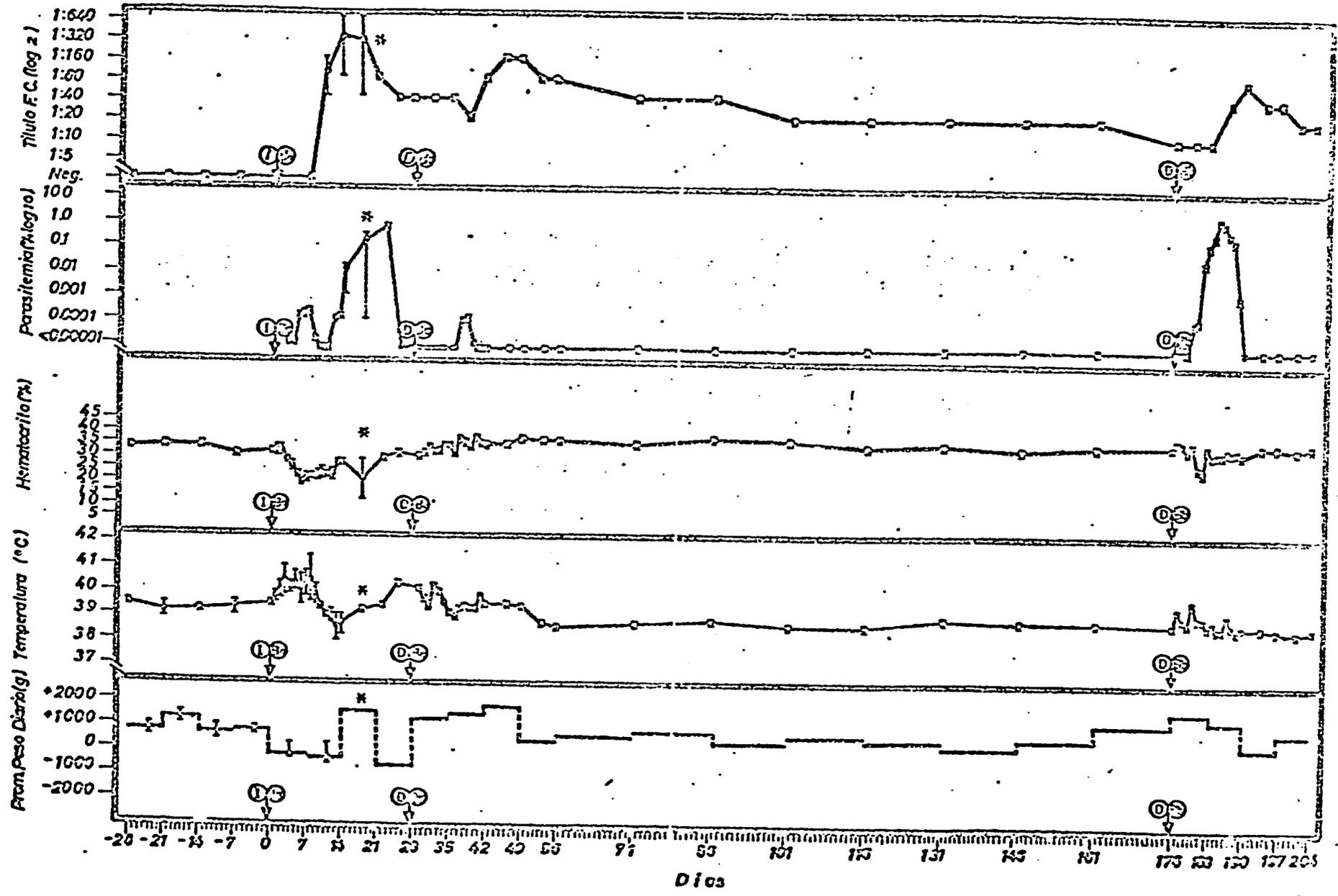


Figura 26. Promedio del título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso diario de 2 terneros inoculados ( I ) ( Bb ) con  $1 \times 10^4$  eritrocitos parasitados por B. bigemina, 4 semanas más tarde con una descarga ( D ) ( Bb ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. bigemina y 4 meses después una segunda descarga ( D ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina. Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.

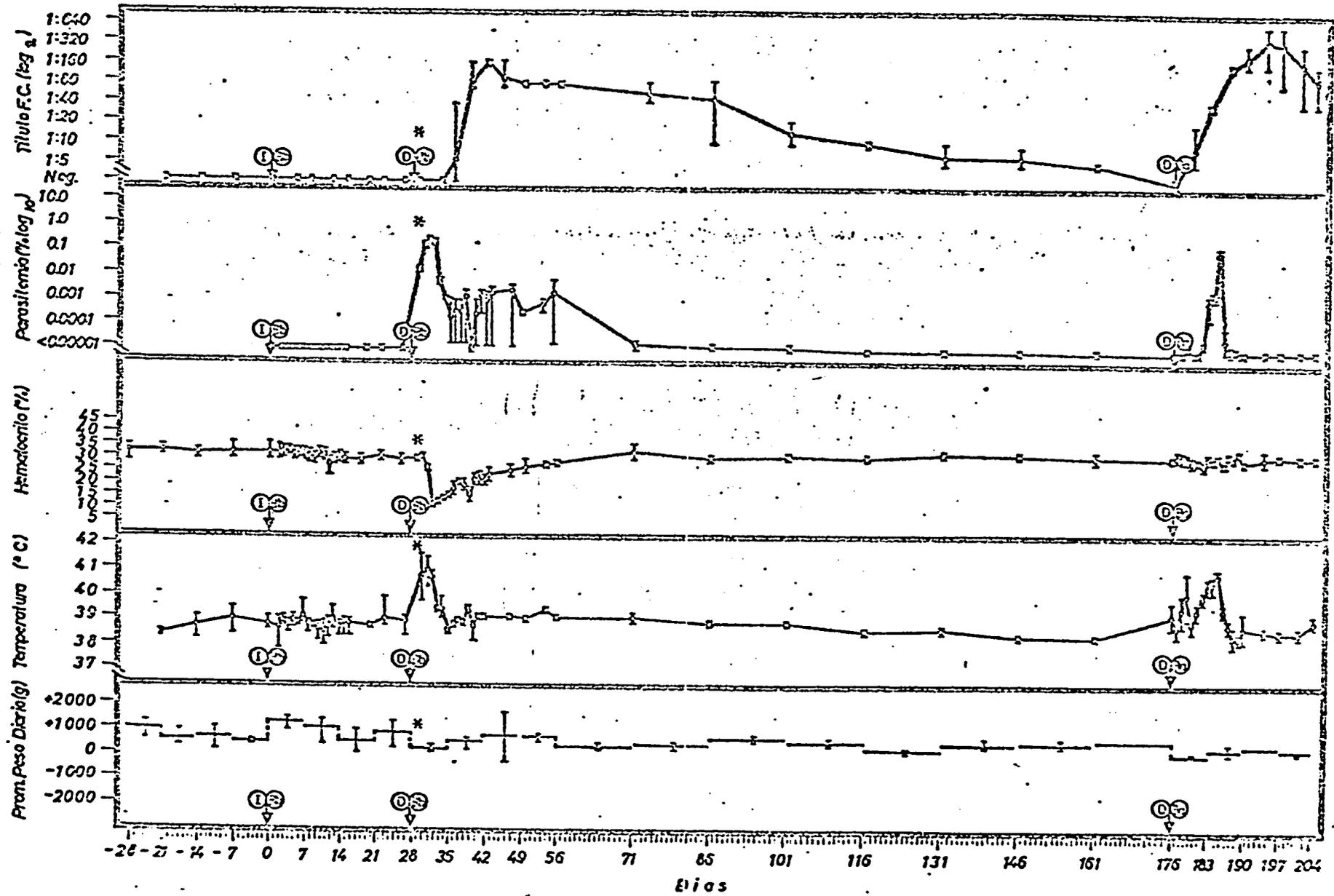


Figura 27. Promedio del título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso diario de 3 terneros inoculados ( I ) ( Bb ) con  $1 \times 10^7$  eritrocitos parasitados por B. bigemina, 4 semanas más tarde con una descarga ( D ) ( Bb ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. bigemina y 4 meses después una segunda descarga ( D ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina. Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.

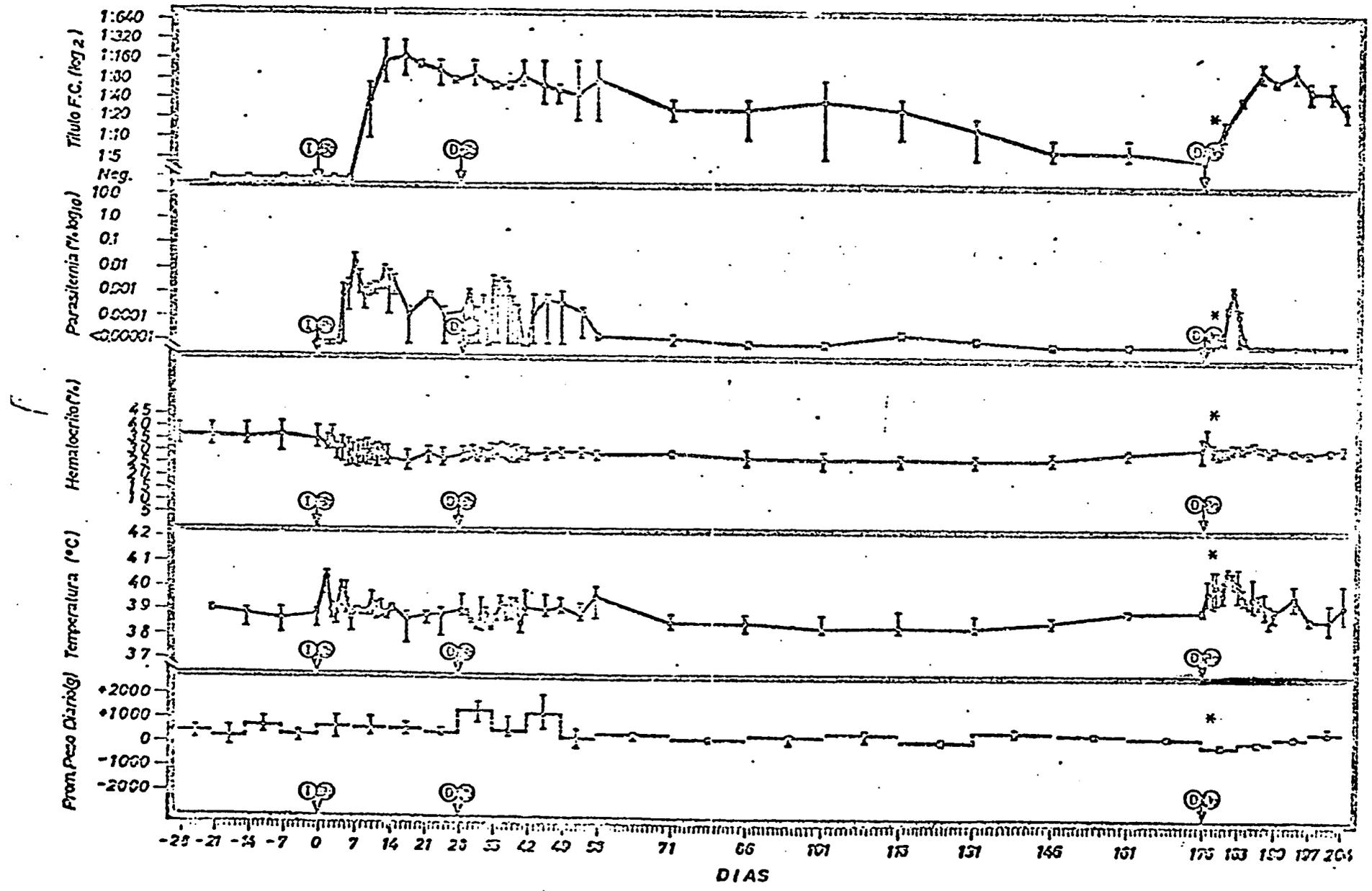


Figura 28. Promedio del título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso diario de 3 terneros inoculados ( I ) ( Ea ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina e inactivados a  $56^{\circ}\text{C}$  por 35 minutos, 4 semanas más tarde con una descarga ( D ) ( Ea ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina y 4 meses después una segunda descarga ( D ) ( Eb) con  $1 \times 10^{10}$  eritrocitos parasitados por B. bigemina. Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.

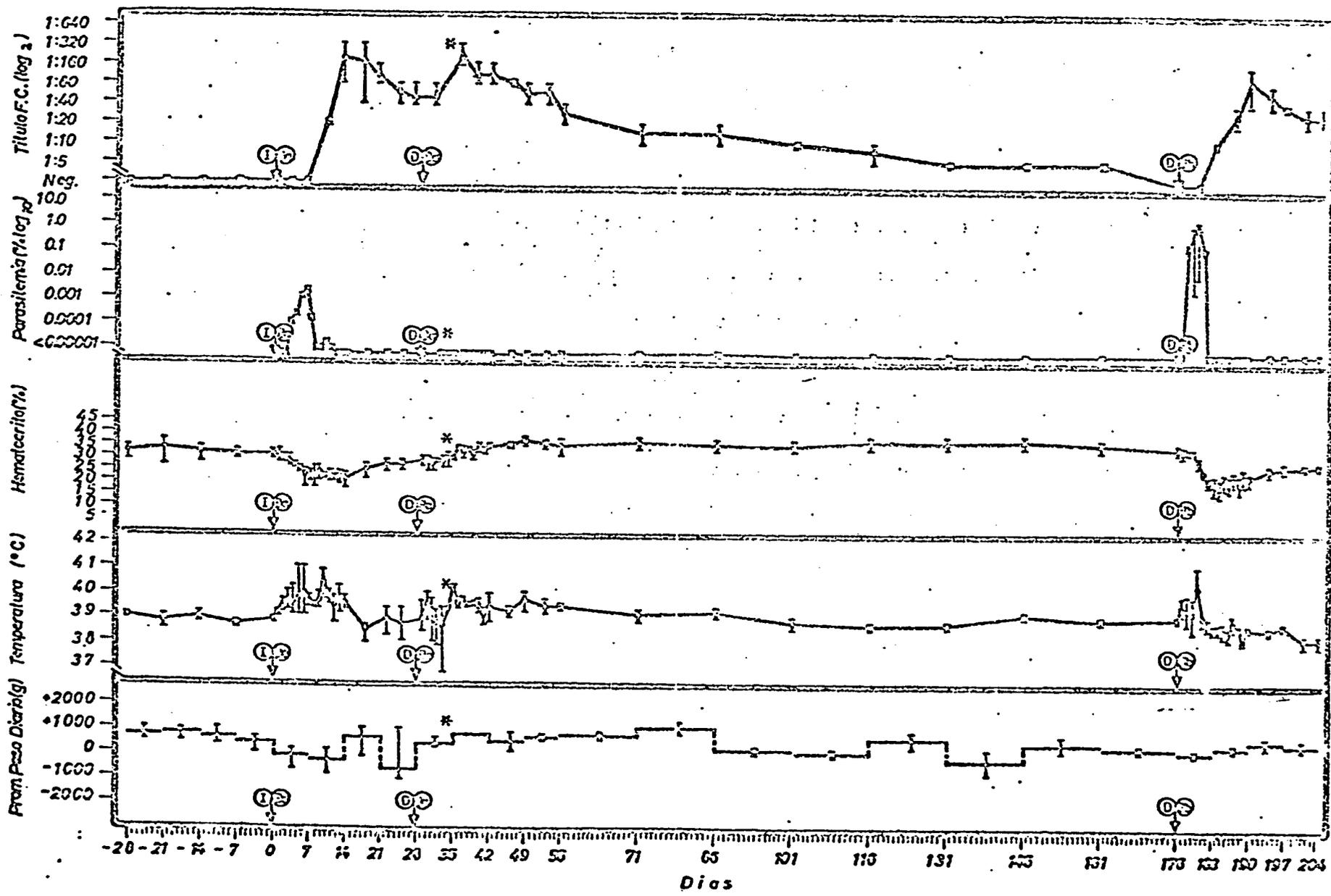
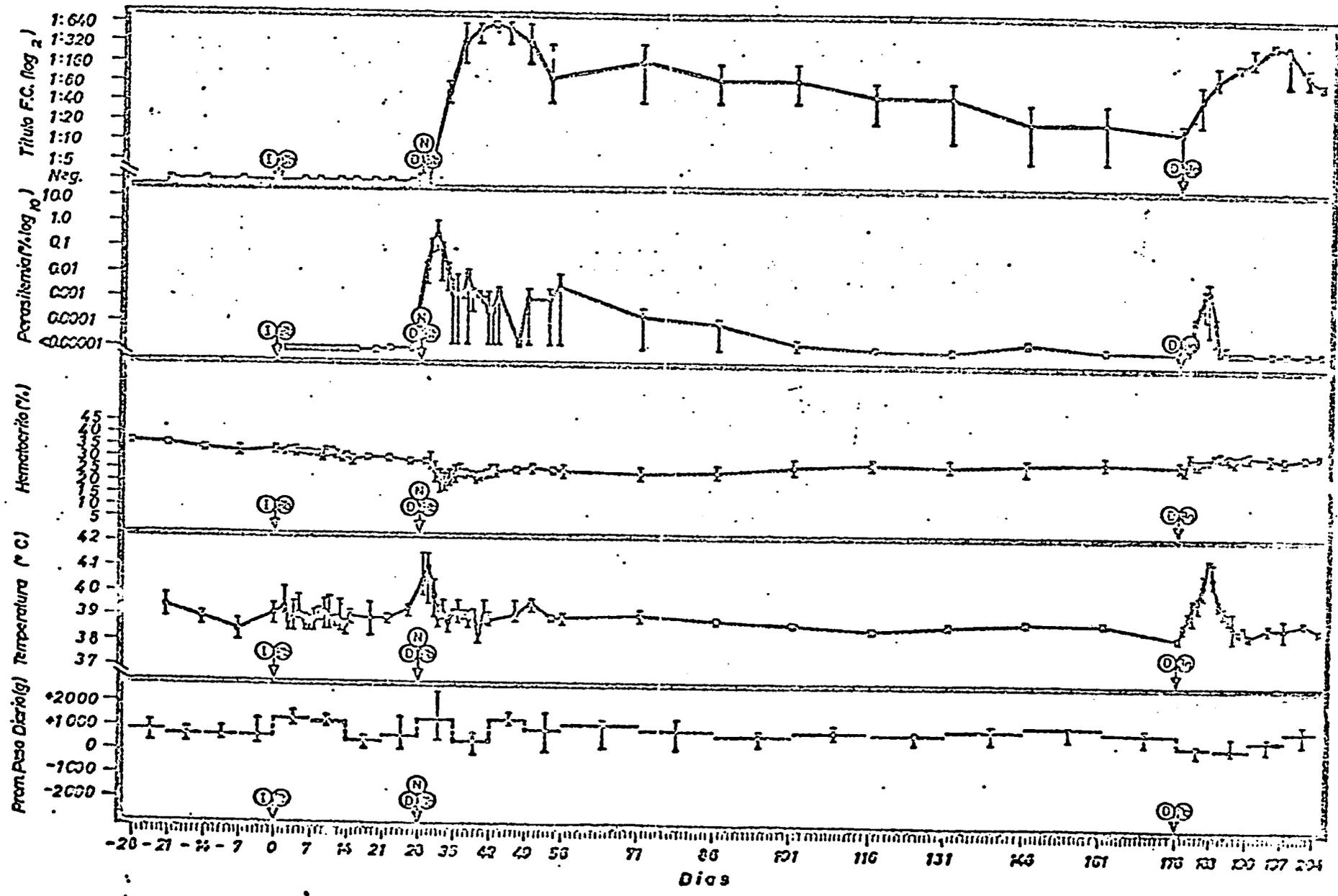
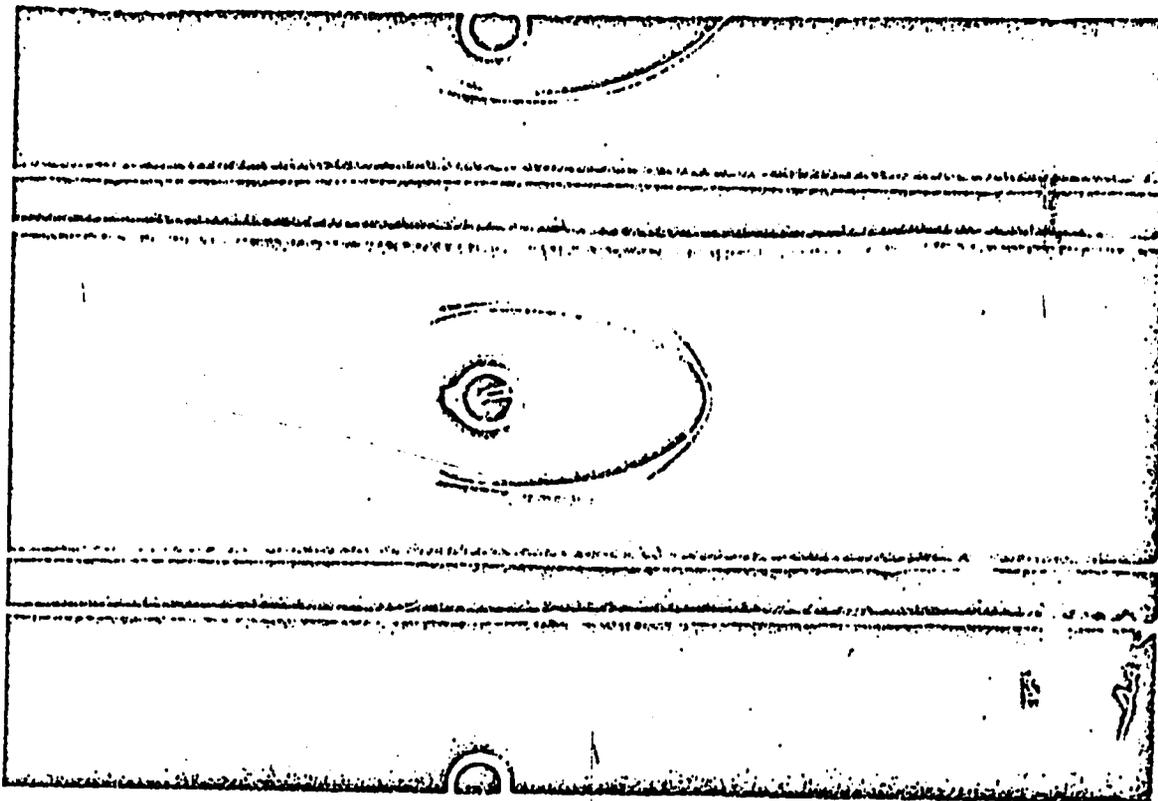


Figura 29. Promedio del título de la Fijación del Complemento, del % de eritrocitos parasitados, del % del hematocrito, de la temperatura rectal y del incremento de peso-diarario de 3 terneros inoculados ( I ) ( Bb ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. bigemina e inactivados a  $56^{\circ}\text{C}$  por 35 minutos, 4 semanas más tarde con una descarga ( D ) ( Bb ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. bigemina y 4 meses después una segunda descarga ( D ) ( Ba ) con  $1 \times 10^{10}$  eritrocitos parasitados por B. argentina. Los 3 terneros fueron negativos hasta el día de la primera descarga ( N ) . Las líneas verticales muestran las fluctuaciones mínimas y máximas de los valores en el parámetro en los días señalados.





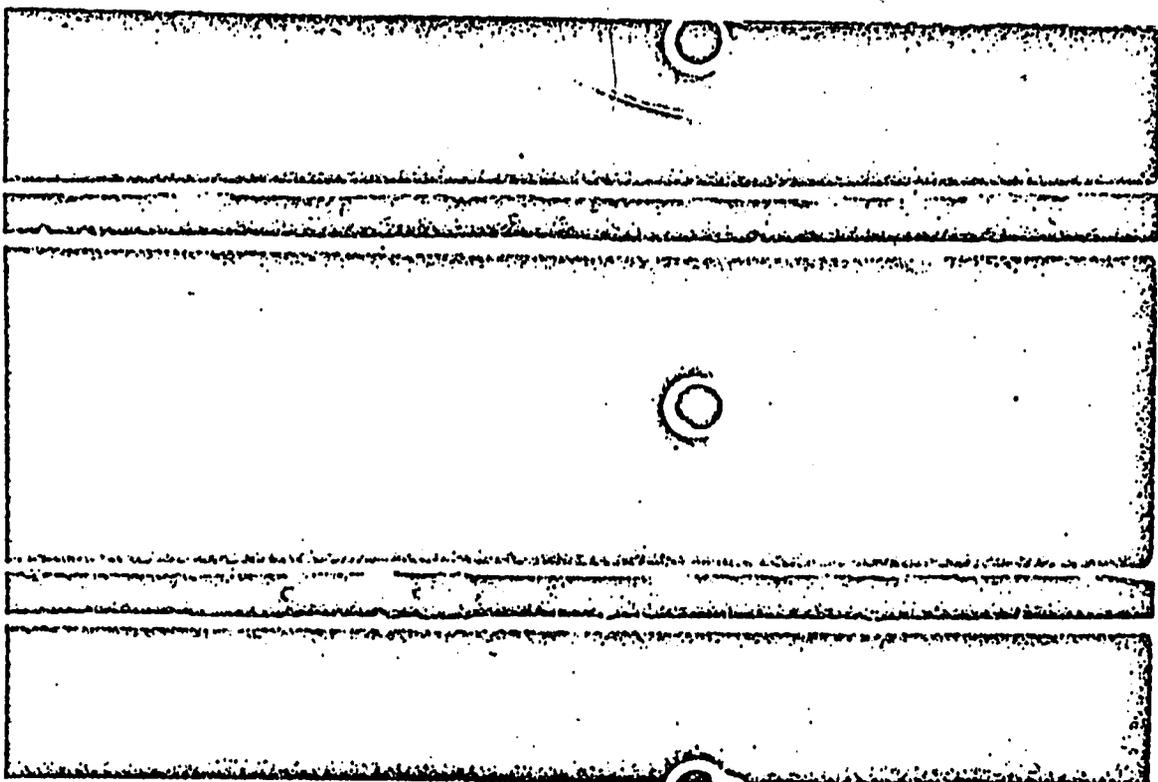
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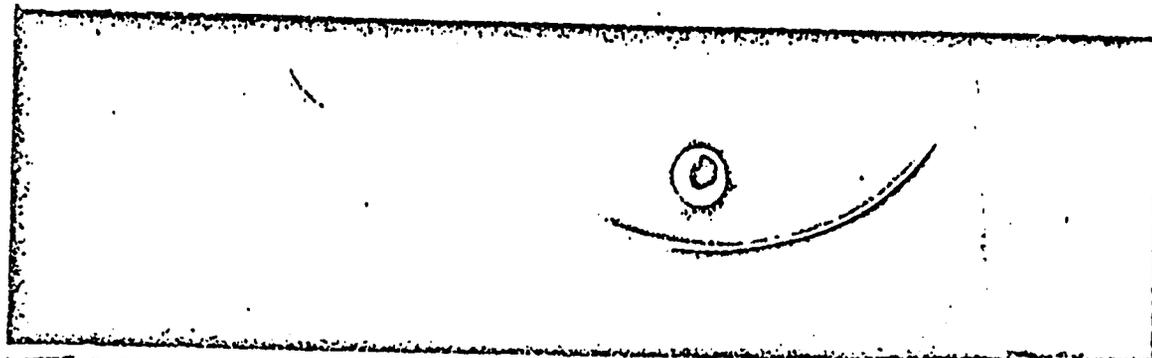
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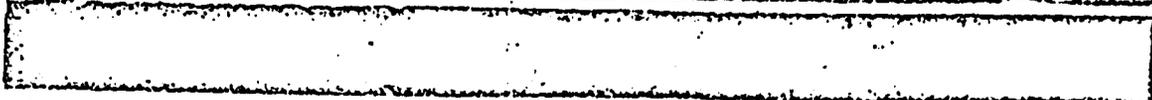
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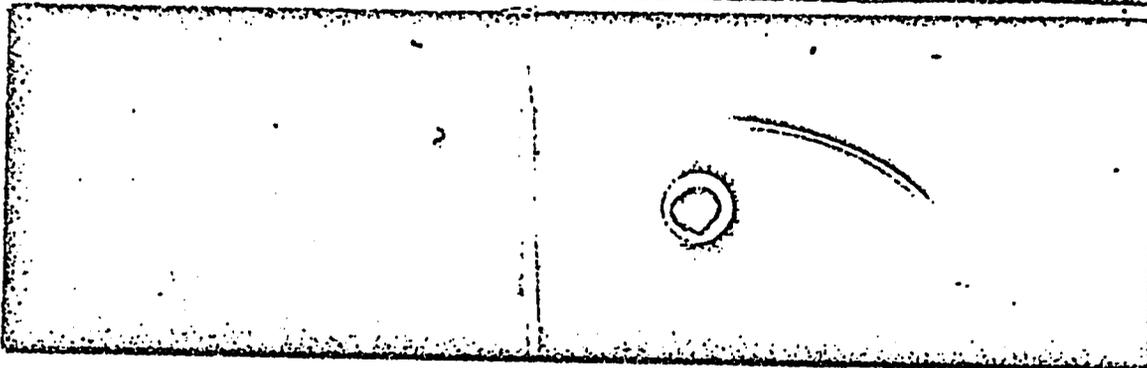
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Suero  
B. argentina



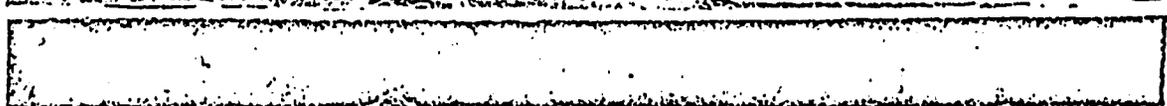
Antisuero  
B. argentina  
/absorvida



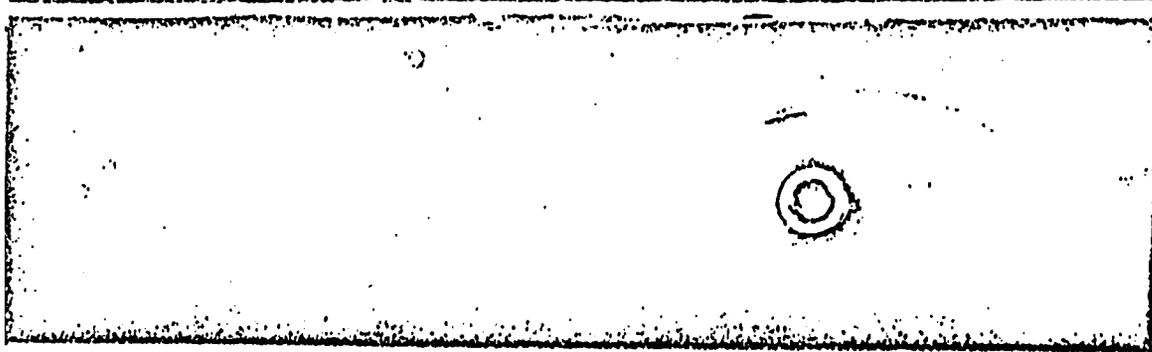
Suero  
B. bigemina



Suero  
B. argentina



Antisuero  
B. bigemina  
/Absorvida



Suero  
B. bigemina

Figure 15

The In Vitro Cultivation of Babesia bigemina Utilizing Bovine Cells in Culture.

INTRODUCTION

The host specificity of B. bigemina limits research efforts due to the use of expensive cattle as experimental animals, and because of the difficulty of interpreting data obtained with a limited number of animals. An in vitro cultivation system would be beneficial to the study of B. bigemina and would contribute to our knowledge of the host-parasite relationship as well as adding important information concerning the life cycle of the parasite in the vertebrate host. Such studies could lead toward more rational methods of prevention, treatment and chemotherapy of bovine babesiosis. An in vitro tissue culture system would be useful for the production of an effective vaccine or as an adjunct to premunition methods (22).. The information acquired and the technics developed during the present project could conceivably be applied to the study of other important Babesia spp.

Tissue culture methods and technics have been applied most commonly with viruses; however, they have also been employed for rickettsiae, mycobacteria, mycoplasma and many parasitic protozoa (17)..

Anaplasma organisms, generally considered of the rickettsiae group, are one example of an intracellular blood parasite grown in vitro. In 1953, Rossi et al. used a procedure to maintain

erythrocytes and was able to demonstrate an initial increase in the percentage of the cells infected with Anaplasma marginale; however, the number and percentage of infected erythrocytes declined over the period of a week (21). In 1966, Zablotskii et al. used Anaplasma ovis infected sheep blood to inoculate monolayers of human liver cells and monkey kidney cells. (32). After 3 days, the number of infected sheep erythrocytes in the culture medium had doubled in number, while the monolayers remained uninfected.

Theileria are intracellular blood parasites which are classified similarly to the genus Babesia. One member of the genus, Theileria parva is the etiologic agent of East Coast Fever in Africa, and has a relatively rigid host specificity. Due to its host specificity, pathogenicity and certain features of its life cycle which are often compared to those of Plasmodium and Babesia, it has stimulated much interest and has been the object of much research activity, such as in vitro cultivation (30). Some of the early culture work by Tsur with Theileria in 1945 resulted in limited survival of the organism up to 18 days without multiplication (23). Attempts to cultivate T. parva in splenic explants in plasma clots were reported by Tsur et al. in 1957 (24). A year later, similar work with Theileria annulata and

T. parva was done by Brecklesby *et al.*, in which schizonts developed in explants of infected spleen and lymph nodes, yet they were unable to transmit the infection to uninfected tissue (2). The first practical use of the in vitro cultivation system was made by Hawkings in 1958, in testing for chemotherapeutic activity of compounds against T. annulata (4).

In 1959, Tsur *et al.* demonstrated the development and multiplication of Koch bodies of T. annulata and T. parva for 6 serial passages in rodent splenic tissue cultures for 2 months (25). They also showed that the 5th passage material was infective for a susceptible calf. The schizonts of T. annulata and T. parva were cultivated by Tsur *et al.* in a monolayer tissue culture system (26). In 1964, the mode of multiplication of the Theileria in cultures of bovine lymphocytic cells was studied by Hulliger *et al.* (7). Additional research on the cultivation of Theileria has been reported (8,27,28,33,34). Other studies were made concerning the transition of developmental stages of T. parva in tissue culture systems at different temperatures (9).

An attenuation of the virulence of T. annulata in tissue culture was shown by Tsur *et al.* and Hooshmand *et al.* (29,6). Malmquist *et al.* made an important contribution in 1970 by the

cultivation of bovine spleen cell lines infected and transformed by T. parva (16). The system was subsequently used as a source of inoculum for immunization against East Coast Fever by Brown et al. (3).

Procedure: The spleen and lymph nodes obtained from Holstein-Friesian calves of either sex, 4-6 months of age, will be used for primary cultures. The calves will be obtained in Colombia, South America, from areas considered hemoparasite-free. Each calf will be examined hematologically for trypanosomiasis, babesiosis and anaplasmosis by Giemsa stained blood smears (11), and serologically for babesiosis and anaplasmosis by complement fixation methods (5,13,1). Also, pooled blood samples from these calves will be subinoculated into susceptible splenectomized calves. Calves found to be free of hemoparasites will be used throughout the experiment as tissue donors and susceptible animals.

Two groups of tissue donor calves will be used, (a) noninfected calves and (b) calves infected with a purified Colombian isolate of B. bigemina. Infection of the calves with B. bigemina will be either by blood inoculation or by natural transmission using singularly infected Boophilus microplus ticks (10,14). The 2 organs will be collected aseptically by

biopsy or at post mortem at the time of peak parasitemia. The cells will then be dispersed by physical means described by Malmquist et al. (15) or by standard enzymatic means utilizing trypsin. The dispersed cells will be suspended in a growth type medium, consisting of Eagle's Minimum Essential Medium with Earle's Salts supplemented with fetal calf serum, non-essential amino acids and vitamins, and inoculated into culture vessels and incubated at 37C. Monolayers of cells obtained will be subcultured at regular intervals depending on the time required to reach confluency. In addition, cells growing suspended in the medium will be subcultured and treated in the same manner as monolayers.

On the 1st, 6th and each 5th subculture thereafter, samples of noninfected cells and infected cells grown on cover slips in Leighton tubes as well as cells growing suspended in the medium will be stained and compared for evidence of infection. Three staining methods will be utilized. The first method will be Giemsa stained slides for the morphology of the cells and parasites (11), with bright field microscopy. The second method will be a vital staining technique using acridine orange (31), with fluorescent microscopy. The third method will be an immunofluorescence technique utilizing either a direct

fluorescent antibody staining procedure described by Ludford in 1969 (12), or an indirect fluorescent antibody staining test using a modification of a method described in 1968 by Ross et al. (20). From the same passage levels, a sample of the cells will be suspended in a growth medium with a protectant added and held under liquid nitrogen until needed in case of contamination or other loss of cells.

A biological assay for infectivity will also be performed on the infected cells at the time of the 1st, 11th and 21st subcultures. A sample of cells will be suspended in a balanced salt solution and used for the subcutaneous inoculation of a susceptible, splenectomized calf. The response of the calf to the inoculum will be assessed in the following manner. The calf will be subjected to daily clinical examination. The rectal temperature will be taken and recorded at daily intervals. Blood samples will be obtained for preparing thin and thick blood smears and stained by Giemsa and acridine orange techniques. Collected serum samples will be titrated for specific complement-fixing activity against B. bigemina. The inoculated calf will be back-challenged on day 28 with infected blood containing  $10^9$  organisms to establish if the calf is resistant to the same B. bigemina as a result of the in vitro inoculum.

## RESULTS

Cell lines, derived from lymph nodes, hemal lymph node, and spleens of experimentally infected and non-infected calves, have been established. First passage 9 hour cell lines derived from infected calves were inoculated into susceptible splenectomized calves and demonstrated to be infective, indicating that the organism survived the tissue processing method. Likewise, the second passage 22 day old cell line derived from infected calves was demonstrated to be infective in susceptible splenectomized calves, indicating that B. bigemina at least survived for 22 days and may have even proliferated. Approximately 15 passages will be done at 2 weeks intervals with further verification of infectivity in susceptible splenectomized calves.

Cell lines derived from normal non-infected calves were inoculated with bovine blood infected with B. bigemina and the organism was observed on Giemsa Stained coverslips covered with the tissue culture material for up to 10 days. In the second passage of this inoculated cell lines as well as that from the cell derived from infected calves, an organism has been observed in 22 to 43 day old cultures. The organism occurs intracytoplasmatically and often impinges on the nucleus of infected cells. Apparently, it first appears as 10 to 18 microns spherical or ovoid vacuolar morula-like structures with a dark basophilic limiting membrane. Later, the morula can be seen to contain several smaller reddish-purple organisms or outlines of organisms. Finally, the morula ruptures and up to about 40 small 2X3 microns banana shaped organisms with a central dark basophilic nucleus and a pale hemophilic cytoplasm can be observed. Subinoculation of these organisms into susceptible splenectomized will be performed

to determine if they are infective. The classification and taxonomy of these organisms remains to be clarified.

PREMUNIZATION OF CATTLE AGAINST BABESIA ARGENTINA WITH INFECTED PARTICLES  
OF TICK ORIGIN

INTRODUCTION

Due to the rapid increase in world population and industrialization of many subsistence agricultural areas, an increase in agricultural production in developing areas of the world is becoming more important. The largest percentage of developing countries is in the tropics. One of the major problems faced by emerging nations is that of access to adequate sources of protein. One source of readily acceptable protein is beef.<sup>22</sup>

Hemotropic diseases have limited the production of beef in tropical and other regions. Babesiosis is one of the most wide spread and costly disease of cattle in the world.<sup>22</sup>

Many investigations have been concerned with immunity to babesiosis. A state of premunition or co-infectious immunity acquired naturally by cattle in enzootic areas<sup>16</sup> or induced by the injection of blood from infected animals has been the most successful approach in the reduction of fatalities from babesiosis.<sup>3, 8, 14, 34, 38</sup>

One of the most successful vaccines against Babesia argentina is that used in Australia,<sup>10, 24</sup> which was developed by Cailow and Mellors and induces premunition against B. argentina by the injection of blood containing approximately  $10^7$  organisms as a standard dose.<sup>7</sup> The administration of this type of vaccine, in the absence of natural tick transmission may have to be repeated.

An undesirable sequela to blood inoculation is the occurrence of neonatal immunohemolytic anemia.<sup>11, 19</sup> Inoculation of bovine blood into cattle has the inherent risk of disease transmission. A further possible disadvantage of blood passaged Babesia is a reduced ability of these organisms to infect ticks.<sup>26, 31</sup> The loss of infectivity to ticks would be an advantage in an

eradication scheme, but, where protection is dependent on re-exposure, it is best obtained by natural tick transmission.

The only non-bovine tissue in which growth and development of the Babesia organism occurs is the tick. The ability of Boophilus microplus to act as a host in which growth occurs, increases the likelihood that concentration and possible increase of antigenic units may occur in tick tissues.

Changes in the virulence of Babesia occur when blood is serially passaged. In as much as development takes place in the tick, it is less likely that changes in virulence of the organism would take place. 17

Tick materials have been injected into cattle in an effort to infect them with babesiosis or other bovine hemoparasites. Success has not been recorded using tick tissue as a source of infection in bovine babesiosis, however, the trials were not entirely conclusive.

Procedure

A strain of Boophilus microplus ticks free of detectable bovine hemoparasites will be obtained from Colombian strains isolated by others. If such ticks are not available, collections will be made of replete female ticks which have fed on animals not known to have been infected with bovine hemoparasites. The hosts must show no evidence of infection with bovine hemoparasites on examination of blood smears and have negative complement fixation tests to anaplasmosis and babesiosis. Proof of non-infectivity will be demonstrated by feeding the progeny of the ticks on susceptible splenectomized calves. If no evidence of hemotropic disease is noted in the host calf after 60 days, the F<sub>2</sub> tick generation will be considered bovine hemoparasites free.

Adult female B. microplus will be infected with B. argentina by feeding them on infected cattle. An alternate method of enabling ticks to ingest parasitized cells is the capillary feeding technique. The ticks will be checked for infectivity by grinding larvae from 1 gm. of eggs (approximately 20,000 larvae) with a mortar and pestle with crushed glass fragments in a medium consisting of Eagle's minimal essential media with Earl's salt solution and bovine serum albumin. The mixture will be allowed to sediment for approximately one hour at 4°C. and the supernate removed by vacuum. The supernate will be injected into splenectomized calves.

The larvae used to determine infectivity will be injected into the calves at the following developmental stages:

1. One week following larval emergence from the egg.
2. Four weeks following emergence from the egg.
3. Four days after commencement of feeding on a non-infected calf.

\*Texas A & M Institute of Tropical Veterinary Medicine.

4. One week after commencement of feeding on a non-infected calf (following larval moult).
5. Two weeks after commencement of feeding on a non-infected calf (following nymphal moult).
6. Four days after drinking bovine serum. 32
7. Four to seven days after commencement of feeding on abnormal hosts, such as rabbits <sup>1,15</sup> guinea pigs, <sup>37</sup> or chicken embryos. <sup>28</sup> Providing that sufficient <sup>6</sup> numbers of larvae can be raised on these hosts.

A number of ticks will be allowed to remain attached to the non-infected calf, to serve as a control of infectivity of the strain of tick used.

A homogenized tick supernate which is successful in infecting cattle will be prepared as indicated earlier and frozen in liquid nitrogen using a diluent of glycerol and bovine fetal serum. <sup>2,9</sup> After being frozen for at least one week, a portion of the supernate will be thawed in a water bath and injected into a splenectomized calf to assure retention of the viability of B. argentina.

The larval antigen will be titrated for infectivity in at least four groups of two calves each. An arbitrary dilution of  $10^{-3}$  will be used as a starting point and ten-fold dilutions will be used, which will be increased or decreased depending on response of the initially infected calves. Titration will be based on the relationship of volume of inoculum and incubation <sup>18</sup> period.

Infection in calves will be determined by; examination of thick and thin blood smears for the presence of Babesia organisms, <sup>20</sup> complement fixation, packed cell volume and temperature variation. The end of the incubation period is considered to be when the calf exhibits a parasitemia on examination of a thick or thin blood smears or by a rise in adjusted morning temperature to 40.5°C. or higher.

If a spread is noted in the length of incubation periods, a regression coefficient will be calculated to see if a correlation exists between the size of inoculum and incubation period. If a significant correlation exists a theoretical infective dose in non-titrated material can be estimated. By use of the regression coefficient based on incubation period, the infectivity of various infected tick tissue can be estimated.

In conjunction with the infectivity trials and titrations, the tick Babesia antigens will be evaluated quantitatively by gel diffusion and complement fixation.  
21

The protective response elicited in cattle by use of viable Babesia antigens from tick tissue hemogenate will be determined. Premunized calves and controls will be challenged by placing 40,000 infected larvae on the back of each animal. Response by the various groups will be based on clinical and laboratory examinations. Data derived from these experiments will be evaluated to determine if field testing would be worthwhile.  
16

## RESULTS

Preliminary results indicate that all attempts to transmit babesiosis with tick origin material have failed, most probably due to the small numbers of infective particles (vermiculi) in the ground up tick supernatant. Further studies are in progress to produce higher Babesia argentina infections of Boophilus microplus ticks. Much higher Babesia infections of the ticks have been obtained and are now being processed and injected into susceptible splenectomized calves to verify infectivity.

A COMPARISON OF THE ANTIGENIC PROPERTIES OF ERYTHROCYTIC BABESIA BIGEMINA  
IN ACUTE AND CHRONIC BLOOD BORNE AND TICK BORNE INFECTIONS IN CATTLE

INTRODUCTION

Immunity induced by parasitic protozoa has many common features with that of other infectious organisms, however, due to the complex life cycles of protozoa, a simple reaction of the host to a single stage of the parasite is not the case. On the contrary, a variety of immunological responses are evoked by the various tissue stages of the organism in the invertebrate host as well as in the vertebrate host by the final products circulating in the blood; in some instances by the continually changing antigenic pattern (Neal et al., 1969).

Curnow (1968) indicated that possibly antigenic variants occurred in one or more strains of Babesia argentina arising by a process similar to that demonstrated by the agglutination test in chronic Plasmodium knowlesi infections (Brown and Brown, 1965). Antigenic variation has not been concretely demonstrated in Babesia. Evidence that different strains of the same species do exist arose because the movement of cattle in Australia from one locality to another were followed by an outbreak of babesiosis in previously immune cattle (Seddon, 1952). This suggests by analogy with trypanosomal and malarial infections that antigenic variation of Babesia after the acute

infection does occur.

With respect to Babesia antigens, the majority of the research has been done on blood borne bovine babesiosis and the minority on the tick borne infections. Such experiments usually provide information applicable to the disease as it occurs in the field, but most of the life cycle of Babesia species occurs in the tick vector (Rick, 1964), thus one is probably examining an altered parasite when studying blood borne infections. It is apparent that such experiments should be confirmed with tick borne infections (Brocklesby et al., 1971).

The purpose of the present study is to determine possible antigenic differences in the acute and chronic blood borne and tick borne Babesia bigemina infections of cattle. It is anticipated that the results of the present research will further aid in the understanding of the mechanism of immunity and possibly show that an antigenic pattern (shift) exists as it does with other Babesia species as described by Sibirinovic et al., (1967) and Ristic et al., (1971). In addition, the antigens may possibly be used in the development of new serologic techniques or vaccines.

## MATERIALS AND METHODS

## Experiment 1. Experimental Animals

A total of 61 four-month-old Holstein-Friesian calves, a mixture of males and females, will be purchased from the Noviciado Farms, Cota, Cundinamarca in the Savannah of Bogota, an area free of Boophilus microplus. At approximately 4 months of age, after insuring they are free from hemotropic infections by utilizing the complement fixation test (CF), packed cell volume (PCV), blood smears and subinoculation, they will be sent by air freight to Turipana. The calves will be experimentally utilized as depicted in Table 1.

Table 1. Number of Experimental Calves with Reference to the Part of Utilization.

Materials and Methods. (Experiment)	No. of calves (Splenuctomized)	No. of calves (Intact)
2- Purification of <u>B. bigemina</u>	6	1
3- Obtain clean <u>B. microplus</u>	5	0
4- Obtain infected <u>B. microplus</u>	3	0
5- Blood borne infection	0	5
5- Tick borne infection	0	5
5- Antigen production (acute and chronic blood and tick borne infections)	4	0
8- Host response to erythrocytic <u>B. bigemina</u> antigens	0	32

Six male and 6 female guinea pigs will be obtained from the colony at Laboratorio de Investigaciones Medicas Veterinarias-Instituto Colombiano Agropecuario, Bogota and transported to Turipana where they will be housed in pens constructed on the floor for later use in experiments 3 and 4.

Twenty New Zealand White rabbits, 3 to 6 months, will be purchased from a local supplier and used for the production of antiserum for serological tests as outlined in experiment 7.

#### Experiment 2. Isolation, Purification and Maintenance of

##### B. bigemina

Babesia bigemina will be isolated from the blood of acutely infected calves which will be exposed to field challenge with infected B. microplus in Turipana. The infected blood will be rapidly passaged through 5 splenectomized calves free of hemotropic diseases (Sergent et al., 1933; Callow and Hoyte, 1961). The purity of the Babesia isolate in the 5th splenectomized calf will be ascertained by subinoculation of the blood into an intact calf and a splenectomized calf which will be monitored weekly for 3 months by the following parameters: CF test, PCV, and blood films (thick and thin). The calves should have a pure infection with B. bigemina without other blood contaminants.

To maintain the isolate for further use, the blood will be collected in sterile 2 l Erlenmeyer flasks using 1.2 g/l

disodium ethylenediaminetetraacetate (EDTA) as an anticoagulant. It will then be mixed with 11.6% sterile glycerol in 100 ml aliquots and incubated for 30 minutes at 4 C. After incubation, the blood will be stored in a REVCO Ultra Low temperature freezer or Dry Ice cabinet at -70 C (Frerichs et al., 1968; Pipano and Senft, 1966).

### Experiment 3. Isolation, Purification and Maintenance of

#### B. microplus

Engorged adult female B. microplus ticks will be collected from adult cattle in Turipana and allowed to deposit eggs which will later hatch to larvae. The engorged females, which deposit approximately 2,000 eggs, will be maintained separately so that the progeny of each individual adult will be known. The 10,000 larvae from each of 5 adult females will be placed on the ears of each of 5 splenectomized calves free of hemotropic diseases, hence, 5 adult females will be screened for the absence of Babesia infection. The splenectomized calves will be monitored weekly for 3 months by CF testing, PCV, blood smears and subinoculation to verify the absence of other hemotropic diseases, at which time, the ticks will be considered to be noninfected.

The noninfected B. microplus ticks will be maintained by feeding on disease free guinea pigs and cattle. Some of the ticks will be kept in desiccators, using different humidities.

produced by various solutions and temperatures in order to maintain them for long periods without feeding (Roby et al., 1964).

Experiment 4. Infection of B. microplus free of B. bigemina

Approximately 5,000 to 6,000 uninfected B. microplus larvae (experiment 3) will be placed on the bodies of 3 splenectomized calves free of hemotropic diseases. The calves will be inoculated intravenously (IV) at nymphal molt with an infection dose ( $1 \times 10^9$  organisms) of purified B. bigemina from experiment 2. The ticks will be collected at the optimal time of infection (last 24 hours of adult repletion) and maintained as described in experiment 3.

Experiment 5. Acute and Chronic Blood Borne Tick Borne Infection

a. Acute blood borne infection

Five intact calves will be inoculated IV with an infection dose of approximately  $1 \times 10^9$  organisms of purified B. bigemina from experiment 2. At the time of maximal parasitemia, the calves will be bled, using EDTA (1.2 g/l) as the anticoagulant and the erythrocytes mixed with 11.6% sterile glycerol and frozen at -70 C. A splenectomized calf will be subinoculated IV with a total of 100 cc pooled from the 5 infected calves. When the splenectomized calf has a maximal

parasitemia, it will be exsanguinated under general anesthesia using EDTA (1.2 g/l) as the anticoagulant, and the blood will be processed as described in experiment 6.

b. Chronic blood borne infection

Four months after infection the calves (experiment 5a) will be bled, using EDTA (1.2 g/l) as the anticoagulant and the blood will be processed in a procedure identical to that described in 5a. Also a splenectomized calf will be subinoculated IV with a total of 100 cc pooled from the 5 infected calves. When the splenectomized calf has a maximal parasitemia, it will be exsanguinated under general anesthesia using EDTA (1.2 g/l) as the anticoagulant, and the blood will be processed as described in experiment 6.

c. Acute tick borne infection

Infected B. microplus larvae from experiment 4 (approximately 10,000 to 40,000) will be allowed to feed on the bodies of 5 intact calves. At the time of maximal parasitemia, the calves will be bled using EDTA (1.2 g/l) as anticoagulant, and their blood will be processed in a procedure identical to that as described in 5a. Also a splenectomized calf will be subinoculated IV with a total of 100 cc pooled from the 5 infected calves. When the splenectomized calf has a maximal parasitemia, it will be exsanguinated under general anesthesia using EDTA (1.2 g/l) as the anticoagulant, and the blood will be processed as described in experiment 6.

d. Chronic tick borne infection

Four months after infection the calves (experiment 5c) will be bled, using EDTA (1.2 g/l) as the anticoagulant and the blood will be processed as described in 5a. Also a splenectomized calf will be subinoculated IV with a total of 100 cc pooled from the 5 infected calves. When the splenectomized calf has a maximal parasitemia, it will be exsanguinated under general anesthesia using EDTA (1.2 g/l) as the anticoagulant, and the blood will be processed as described in experiment 6.

Experiment 6. Isolation and Purification of the Babesia  
Erythrocytic Antigens

The method of Mahoney (1962b, 1967) for the preparation of the Babesia erythrocytic antigens will be used. Blood will be drawn from the jugular vein of the B. bigemina infected splenectomized calves with high parasitemias (experiment 5) into sterile 2 l Erlenmeyer flasks using EDTA (1.2 g/l) as the anticoagulant. The plasma will be removed after centrifugation and the erythrocytes will be washed twice in sterile physiological saline. The volume of packed erythrocytes will be measured and 10 times this volume of 0.35% sodium chloride at 4 C will be added. Lysis will be allowed to proceed for 1 hour at 4 C. The material will then be centrifuged at 5,000 g for 30 minutes at 4 C. After centrifugation, the supernatant fluid will be saved for further testing, and the

sediment, resuspended in sterile physiological saline, will be used to prepare the Babesia erythrocytic antigen extract. Distilled water extracts will be prepared by mixing 1 volume of parasite suspension (sediment) with 2 volumes of distilled water. This preparation will be shaken vigorously for 1 to 2 minutes, and then centrifuged at 5,000 g for 30 minutes at 4 C. The supernatant fluid will be collected and then the above procedure will be repeated 6 to 8 times. The supernatant fluid will be tested for antigenic activity by the slide agar-gel precipitation test (Ristic and Sibinovic, 1964) and then frozen rapidly to -70 C in 2 ml amounts and stored at this temperature until used. The antigen stored by this method should retain its antigenicity for at least 6 months (Curnow and Curnow, 1967). On the day of further processing, the material will be thawed rapidly by immersing the vials in a water bath at 37 C.

Purification by means of molecular sieving (Sibinovic et al., 1968) and/or sucrose density gradient centrifugation (Brakke, 1953) will be attempted. The babesia erythrocytic antigen mixture will be passed through a 1 by 20 cm Biogel P-200 column from which 1 to 2 ml samples will be collected. The pooled Babesia erythrocytic antigen concentrated by the Biogel columns will be further purified by means of density gradient centrifugation technique. Fifty ml sucrose gradients (10 to 30%) will be prepared in cellulose nitrate centrifuge tubes.

Two ml of the Babesia erythrocytic antigen mixture will be layered on to the gradient and centrifuged at 75,000 g at 4 C for 12 hours, in an ultracentrifuge. Aliquots of 5 ml's will be withdrawn through the sides of the tubes at different levels using a small guage needle and a syringe. Each sample will be dialyzed against 0.85% saline for 72 hours to remove the sugar. The sample will be tested for antigenic activity. The slide agar-gel precipitation test will be used to determine the presence of antigens using antiserums from clinically recovered calves. Control tests will employ the preinoculation serums of the same calves. In addition, the test will be used for the detection of serum antibody in the experimental calves.

The Babesia erythrocytic antigens will also be characterized by disc and/or agar-gel electrophoresis (Ornstein, 1964; Davis, 1964; Todorovic, 1967); direct hemagglutination (Butler, 1963; Stavitsky, 1954; Curnow, 1968), and the CF test (Mahoney, 1962b).

#### Experiment 7. Preparation of Specific Antiserum

Five New Zealand White rabbits will be utilized per each antigen. Three to 7 mg/ml of concentrated Babesia erythrocytic antigen plus an equal amount of Freund's complete adjuvant will be mixed for 10 minutes and inoculated intradermally (1 ml/rabbit) in the axillary and inguinal portion of the legs and the abdominal skin of the rabbits with a 23

guage needle. After 5 inoculations (day 0, 15 and 22 with adjuvant and day 28 and 38 without adjuvant) the rabbits will be bled (10 cc) from the marginal ear vein on day 45 and the serum will be tested for the presence of specific antibodies by the slide agar-gel precipitation test.

Experiment 8. Host response to the Babesia Erythrocytic  
Antigens

Thirty-two intact calves will be divided into 16 groups of 2 and inoculated and challenged as described in Table 2.

The parameters to be used to measure the response are temperature rise, PCV, CF, and blood smears.

Table 2. Experimental Design for Homologous Inoculation and Homologous and Heterologous Challenge with Viable Babesia bigemina Isolated from Acute and Chronic Blood Borne and Tick Borne Infections in Cattle.

Inoculation ( $1 \times 10^9$ organisms)	Challenge ( $1 \times 10^{10}$ organisms at 4 weeks)
8 calves-viable <u>B. bigemina</u> from acute blood borne infection (5a)	2 calves-viable <u>B. bigemina</u> from acute blood borne infection (5a)
	2 calves-viable <u>B. bigemina</u> from chronic blood borne infection (5b)
	2 calves-viable <u>B. bigemina</u> from acute tick borne infection (5c)
	2 calves-viable <u>B. bigemina</u> from chronic tick borne infection (5d)
8 calves-viable <u>B. bigemina</u> from chronic blood borne infection (5b)	8 (4 groups of 2) calves will be challenged as depicted in table 2
8 calves-viable <u>B. bigemina</u> from acute tick borne infection (5c)	8 (4 groups of 2) calves will be challenged as depicted in table 2
8 calves-viable <u>B. bigemina</u> from chronic tick borne infection (5d)	8 (4 groups of 2) calves will be challenged as depicted in table 2

## RESULTS

Preliminary results are given in the attached table.

Acute blood-borne babesiosis induced more relapses, a higher parasitemia and higher CF titers. Acute tick-borne babesiosis caused decreased weight gains, a lower hematocrit and higher body temperatures.

Although this project is not completed, it would suggest at this time, that should there be a difference or variation within the same species of organism that the production of a reliable vaccine, must incorporate all types of the same species of blood parasites.

A comparison of the antigenic properties of erythrocytic Babesia bigemina on Acute and Chronic Blood Borne and Tick Borne Infections in cattle.

	BLOOD BORNE	TICK BORNE	DAYS
Recrudescence (#)	3.2	2.4	54
Weight (lbs)	402	378	54
Ave. Min. Packed Cell Volume (%)	24	19	7
Complement Fixation Titer	1/60	1/80	7
Ave. Max. Parasitemia (%)	0.44	0.05	2
Ave. Max. Temperature (°C)	40.4	41.6	2

DEVELOPMENT OF A SPECIFIC AND SENSITIVE INDIRECT FLUORESCENT ANTIBODY TEST  
FOR THE DETECTION OF TRYPANOSOMA VIVAX INFECTIONS

INTRODUCTION

In 1961 Wells et al. (14) conducted field studies in Colombia utilizing standard methods of trypanosome detection (SMTD) in an attempt to determine the prevalence of T. vivax in the Colombian departments of Córdoba, Huila and Valle. The total number of cattle studied was 608 distributed throughout 37 farms in 14 departments. The bulk of the investigation was centered in Córdoba where 17% of 248 cattle examined were positive for T. vivax via the stained smear technique. This technique is only reliable in detecting T. vivax infections in animals with high parasitemias. The number of cattle examined in other locations ranged from 30 to 1. Although the Wells' et al. publication gives some idea of the prevalence and distribution of T. vivax, more extensive surveys are required before an accurate assessment of the importance of T. vivax in Colombia can be made.

The development and application of a sensitive and specific IFA technique capable of detecting acute

and chronic T. vivax infections will greatly facilitate further epizootiological studies. Combined use of the IFA technique with SMTD will ascertain the status of bovine trypanosomiasis due to T. vivax in a herd quickly and effectively (2, 16).

The first successful utilization of the IFA test for the detection of trypanosome infection was made by Fife(5) in the diagnosis of Chaga's disease. Sadun in 1963 (11) modified Fife's technique and successfully used trypanosomes in dry blood smears as antigen. Using the method devised by Anderson (1) to extract serum from dry blood samples, Sadun also demonstrated that such samples collected on filter paper could be used in IFA screening tests for human trypanosomiasis. The same technique involving dried blood samples was successfully implemented by Baily in 1967 (2) who utilized a less involved method of extracting serum from the samples. The use of dried blood samples eliminates the need for rapid transport of refrigerated samples from remote areas to central IFA equipped laboratories.

Cunningham in 1966 (3) and Wilson in 1967 (15) applied the IFA test to the detection of bovine trypanosomiasis. The principal difficulties encountered thus far have been the assessment of the degree of

fluorescence and degrees of cross reaction (9). Mwambu (10) in a 1969 East African survey employed the IFA test as developed by Cunningham in 1966 on 1,406 bovine serum samples. Of 57.5% of the samples considered positive (greater than +3 fluorescence) only 1% exhibited maximum fluorescence. He did not distinguish between T. vivax and Trypanosoma theleri.

The problem of non specificity was adequately illustrated by Weitz in 1963 (13). Using a direct fluorescent antibody technique with conjugated antisera prepared by inoculating rats with Trypanosoma brucei and T. vivax killed organisms, he observed more intense fluorescence with the heterologous antigen than with the homologous antigen.. However, when specific exoantigens were used to produce antisera, cross reactivity did not occur.

The specificity and sensitivity of various antigen preparations with respect to known anti T. vivax sera and sera obtained from cattle singly infected with T. theleri, Trypanosoma evansi, Anaplasma marginale, and Babesia spp. will be compared. These antigen preparations will include intact T. vivax organisms derived from sheep and cattle in addition to exoantigen and soluble antigen of T. vivax origin absorbed to

sheep erythrocytes. Criteria to be used will include the absence or presence and degree of fluorescence obtained in an IFA system.

Following the establishment of a specific T. vivax IFA technique, a serological survey will be conducted of Colombian cattle employing both screening and quantitative IFA techniques (16).

## PROCEDURE

## Development of the Indirect Fluorescent Antibody Test

Trypanosoma vivax initially will be obtained from actual bovine clinical cases and produced by injection of organisms into hemoparasite free sheep and cattle. T. vivax will be identified by morphology, characteristic movement in wet mount, non-infectivity for laboratory mice and culture characteristics. The isolate will be initially passed through a sheep to eliminate possible Babesia spp. The sheep will also be concurrently treated with oxytetracycline I-V at the rate of 12mg/kg for 12 days to eliminate A. marginale. The isolate will then be serially passaged in hemoparasite free sheep and calves until suitable levels of parasitemia are achieved.

Blood smears will be made on 1 x 3" glass slides from blood obtained from sheep and calves all with high parasitemias. The slide preparations will be fixed by acetone, methanol or heat, labelled and stored dry at -20°C until used.

Blood from sheep and calves with high parasitemias will be collected and the trypanosomes separated by one or a combination of the following methods:  
differential centrifugation, hemagglutination (17)

and anion exchange (8). Resulting pure trypanosome suspensions from each species will be fixed by heat on glass slides, labelled and stored dry at  $-20^{\circ}\text{C}$  until used.

Exoantigen will be obtained from the sera of rats and goats with high parasitemias according to the method described by Weitz (13). The sera containing soluble exoantigen will be treated with desiccated rabbit or sheep liver powder to remove heterophillic antibodies. Tanned and/or formalized sheep erythrocytes will be used to absorb the exoantigen from the sera according to methods described by Gill (6, and 7). Slides containing antigen-erythrocytes will be prepared, fixed by physical or chemical means, labelled and stored dry at  $-20^{\circ}\text{C}$  until used.

In addition, a soluble antigen will be prepared by grinding or sonically disrupting whole T. vivax organisms and extracting the soluble antigen with physiological saline. The soluble antigen will then be absorbed with tanned and/or formalized sheep erythrocytes. Slides will be prepared and stored as above.

A standard IFA technique utilizing rabbit anti-bovine globulin tagged with fluorescein isothiocyanate will be used. Suitable controls will be employed.

Reactions will be graded positive or negative. Photographic records will be made.

Sensitivity of the IFA test will be demonstrated by titering known positive T. vivax antisera obtained from calves used in an earlier pathogenesis study of T. vivax in Bogota (4).

Specificity of the IFA test will be demonstrated by treating the above antigen preparations with various dilutions of antisera obtained from cattle singly infected with each of the following organisms: T. theileri, T. evansi, A. marginale and Babesia spp.

Trypanosoma theileri antisera will be obtained from Texas cattle assumed free of Babesia spp., T. evansi and T. vivax by virtue of geographic location, and certified free of A. marginale via the CF test.

Antisera singly specific for A. marginale and Babesia spp. will be obtained from the serum bank in Bogota.

Anti T. evansi serum will be produced by infecting three hemoparasite free calves. Trypanosoma evansi isolate will be obtained from a clinically ill horse and cultured in the laboratory by injection of white rats with infected horse blood.

The antigen preparations described above, con-

sisting of whole intact T. vivax organisms as well as sheep erythrocyte absorbed exoantigen and soluble antigen will be treated with all of the prepared specific antisera. The fluorescent response obtained using a standard IFA technique will be recorded and graded negative or positive. The antigen preparation exhibiting the highest degree of sensitivity and specificity as determined by inspection, will be selected for use in a serological survey.

#### Prevalence and Incidence Surveys

Approximately 2000 serum samples obtained from various regions of Colombia are currently stored at the Instituto Colombiano Agropecuario laboratory in Bogota. Pending the successful development of a specific IFA test for T. vivax, a survey of these serum samples will be undertaken. The minimum serum dilution to avoid non specific fluorescence will be used. Positive samples will be tabulated on a geographical basis.

Field investigations of suspected T. vivax outbreaks will be conducted in order to determine the degree of herd prevalence. Involved herds will be investigated during the acute episode and three to six weeks later.

One or more of the following techniques will be employed in field studies: wet mounts, thick smears, capillary tube technique, the IFA qualitative test using dry blood samples and the IFA quantitative test.

Titers of positive serum samples will be determined and compared to the results obtained from blood samples collected onto filter paper and allowed to dry.

## RESULTS

Preliminary results from using 1) Acetone: Methanol - 60:40  
2) Heat 5 min. at 70°C. 3) Acetone 4) Ethanol 5) Formalin - 1-10%  
6) Methanol as fixatives for T. vivax on glass slides indicated that  
Acetone: Methanol or Heat were far superior to the other fixatives  
for the fluorescent antibody test. If a commercial conjugate (SYGCO)  
was used at a 1:32 titer with a 1:30 known positive anti - T. vivax  
serum, the test resulted highly positive when compared to Luffer and  
negative serum controls. No cross reactions with T. theileri occurred,  
however, further cross reaction testing will be done with T. evansi,  
B. bigemina, B. argentina and Anaplasma marginale. The best antigenic  
preparation of T. vivax for the IFT has been the first calf to rat  
passage using the first high ascending rat parasitemia to avoid the  
immune complex problem. Five rat passages have been accomplished, with  
sheep serum and cortisone supplements, and the inoculum of T. vivax  
was passaged from a calf to a sheep to a calf to the rat. The rats  
had low parasitemias within 2 to 3 days post inoculation. Antigenic  
T. vivax derived from splenectomized calves with cortisone therapy  
were the next best source of antigen for the IFT.

The isolation of the specific T. vivax exoantigen has had numerous  
difficulties primarily due to the lack of quantity of antigen available  
and contamination with serum gamma globulin, however efforts are now in  
progress to produce high rat primary ascending parasitemias to avoid  
this problem.

A FIELD STUDY SURVEY OF THE PREVALENCE INCIDENCE AND ETIOLOGY OF  
 "SECADERA" (A CHRONIC EMACIATING SYNDROME OF CATTLE) IN THE EASTERN  
 PLAINS (DEPARTMENT OF CAQUETA) OF COLOMBIA

INTRODUCTION

At the request of the Colombian Agrarina Reform Agency (INCORA), the Texas A & M group was asked to investigate a chronic wasting disease of cattle suspected to be due to blood parasites called "Secadera" which occurs in the eastern plains of Colombian in the department of Caquetá.

PROCEDURE.

Four hundred and seventy four cattle, including newborns through adults, were examined and 27 parameters were measured of each animal. The following parameters were measured by routine methods: breed, sex, age, general physical status, hematocrit, Anaplasma marginale parasitemia and CF titer, Babesia bigemina parasitemia and CF titer, B. argentina parasitemia and CF titer, Trypanosoma vivax parasitemia, Brucella abortus titer, Leptospira spp. titer, egg or larval counts of Haemonchus spp., Trichostrongylus spp., Ostertagia spp., Cooperia spp., Oesophagostomum spp., Bunostomum spp., Dictyocautus viviparus, Chabertia spp., Trichuris spp., Fasciola hepatica, Moniezia spp., Eimeria spp., tick counts; use of salt, use of minerals, frequency of use of salt and minerals and assessment of management practices other than those listed above. The cattle were divided by age into 3 groups as follows: Group I - 0 to 6 months; Group II - 7 through 18 months and Group III - 19 months and upward.

## RESULTS

In the Group I, multiple regression analyses revealed no significant relationships, however, in Group II, a highly significant relationship between the general physical state of health and the complex composed of blood parasites, internal parasites, salt, minerals, frequency of use of salt and minerals, and management was found. In Group III, multiple regression analyses demonstrated a highly significant relationship between the general physical state of health and (1) blood parasites and breed (2) blood parasites, ticks and breed (3) blood parasites, internal parasites and breed (4) blood parasites, salt, minerals, management and breed (3). "Secadera", the chronic wasting disease of cattle, is due to a complex of several disease causing agents and malmanagement practices of which blood parasites are on the foremost, therefore, control measures would have a large impact on increasing beef production in this tropical zone.

## FUTURE RESEARCH PROJECTS

The project in Colombia will pursue the current experiments and attempt to terminate them and apply their results to future research. Another field trial will be executed at Monteria to evaluate improved premunition techniques which are under investigation presently. An attempt will be made to develop a standard dose inoculum and treatment for A. marginale and B. argentina premunition methods. The IFAT technique will be applied to evaluate the prevalence of T. vivax infections of Colombian cattle. Attempts will be continued to adapt T. vivax to rats without ovine serum and cortisone supplements. Tissue culture research will be continued for the cultivation of B. bigemina in mammalian cell lines and more emphasis will be placed on the cultivation of B. bigemina in Rhipicephalus microplus tick tissue culture systems. Increased emphasis will be given to the study of tick vectors, as to their role in the transmission of babesiosis and anaplasmosis, the life cycle of the ticks, and development of Babesia and Anaplasma organisms in the tick. The effect of Melinis minutiflora (Molasses Grass) pasture on the development of Rhipicephalus microplus and the incidence of bovine babesiosis will be investigated in Carimagua, los Llanos Orientales. Chemoprophylaxis and chemotherapy trials for anaplasmosis and babesiosis will be continued. Species antigenic variation and strain immunity of Colombian isolates will be studied and compared for future application to vaccine production methods. Pathogenesis studies will be performed in calves infected with B. argentina and T. vivax.