

OBSERVATIONS ON THE BLOOD ALTERATIONS ASSOCIATED WITH
EPERYTHROZONOSIS AND ANAPLASMOSIS IN SHEEP
FOLLOWING SPLENECTOMY

by

ARTHUR HARLAN BECKER

D. V. M., Kansas State University
of Agriculture and Applied Science, 1962

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Pathology
School of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1962

TABLE OF CONTENTS

INTRODUCTION	1
REVIEW OF THE LITERATURE	1
History	1
Etiology	4
Incidence	6
Pathogenesis	8
<u>Anaplasmosis</u>	8
<u>Eperythrozoonosis</u>	11
Transmission	13
Effects of Splenectomy	14
Applications of Electrophoresis	17
MATERIALS AND METHODS	20
EXPERIMENTAL PROCEDURES AND RESULTS	23
DISCUSSION	39
SUMMARY	41
ACKNOWLEDGMENTS	44
BIBLIOGRAPHY	45

INTRODUCTION

The diseases eperythrozoonosis and anaplasmosis have been known to occur in sheep for some time, anaplasmosis having been reported by DeKock and Quinlan (1926) in South Africa (48), followed by descriptions of eperythrozoonosis by Neitz, et al. (1934) also in South Africa (45).

Until recently, these two diseases were not known to occur in sheep in the United States (31), (32), (62), typical symptoms being attributed to other causes. The increased incidence of the two diseases in sheep in the United States, especially in animals splenectomized for experimental purposes, suggests that the infections are more common than usually considered due to the subclinical nature of the diseases in most non-splenectomized hosts.

This study is an attempt to determine the incidence of eperythrozoonosis and anaplasmosis in a group of twenty-five adult sheep, and to observe any hematological changes produced by relapse infections with either or both of these diseases in the carrier-infected sheep following splenectomy.

REVIEW OF THE LITERATURE

History

According to Lotze (36) the disease known as anaplasmosis was first described as a mild type of piroplasmosis in cattle by Smith and Kilborne (1893), and it was not until 1910 that it was shown to be a separate disease entity by Theiler in Africa. DeKock and Quinlan, as cited

by Splitter (61), considered the report of Domizio (1919) to be the first authentic description of anaplasmosis in animals other than cattle.

Breed, et al. (6) credit Lestoquard (1924) with the discovery of Anaplasma ovis. Gilbert (26) reported a disease known as "Safra" or yellow disease occurring in sheep and goats in Palestine. Symptoms described were icterus, anemia, dullness, anorexia, rapid breathing, and a rise in temperature. Blood films demonstrated anaplasms similar to Anaplasma marginale of cattle, and anisocytosis was a typical finding.

In 1926, DeKock and Quinlan, according to Rees (49), were the first to discover anaplasmosis in sheep through their study of the effects of splenectomy on cattle, sheep, and horses. Their findings were later summarized in a report by Quinlan, DeKock, and Marais (48) describing the course of the disease in sheep. Jensen (32), early in 1955, stated that a suspected case of anaplasmosis in a flock of sheep in Utah was probably due to some other cause, based on the fact that sheep in the United States were not considered susceptible to clinical anaplasmosis. He attributed the appearance of so-called marginal bodies in the erythrocytes to the result of anemia and subsequent regeneration of erythrocytes. Later that same year, Splitter and co-workers (62) reported anaplasmosis in sheep in the United States for the first time. Further studies on the identity of this species of Anaplasma by Splitter, Anthony, and Twiehaus (61) led to the conclusion that the organism was identical to Anaplasma ovis as described by the workers in South Africa.

Eperythrozoonosis was first described as a parasitic condition occurring in splenectomized mice by Schilling in 1928, according to Neitz, et al. (45). Although at that time there was no conclusive evidence for the acceptance of the small ring-shaped organisms as a

parasitic entity, the transmission from infected to susceptible mice and the chemotherapeutic influence of neosalvarsan in sterilizing the blood was thought to be significant. In 1934, Adler and Ellenbogen (1) reported the finding of several new blood parasites in cattle which they felt had never been observed in ruminants prior to that time; one of these parasites was an Eperythroozoon found in the blood of a splenectomized calf. The name Eperythroozoon wenyoni was proposed for the new parasite in honor of Dr. C. M. Wenyon of the Wellcome Bureau of Scientific Research. One month after the published work of Adler and Ellenbogen, Neitz et al. (45), while conducting experimental studies on heartwater disease in South Africa, reported observing peculiar ring-shaped bodies 0.5 - 1.0 micron in diameter in blood smears from splenectomized sheep. These peculiar organisms were reproduced by inoculating other sheep with the infected blood, and the name Eperythroozoon ovis was proposed for what was believed to be a previously undescribed species of Eperythroozoon. Although the organism had probably been observed previously on many occasions with little attached significance, the description of Neitz and his associates appears to be the first account of eperythroozoonosis in sheep. Later, Neitz (43) further described Eperythroozoon ovis as a blood parasite of sheep capable of producing a mild to a marked degree of anemia in the host. He reported difficulty encountered in the study of the condition due to the fact that 15-20% of available experimental sheep harbored a latent infection of the parasite. The first account of eperythroozoonosis in sheep in the United States was published by Jensen (31) in 1943, although the disease was noted by Lotze (37) to occur in cattle in the United States prior to that time. The fact that Jensen (31) was successful in locating

Eperythrozoon ovis in the first flock of sheep sampled indicates the disease was already probably widely disseminated in the United States.

Etiology

Much disagreement has existed in the classification of the etiological agents involved in anaplasmosis and eperythrozoonosis (10). Neitz, et al. (45) proposed placing Anaplasma and Eperythrozoon both in the family of Anaplasmae under the order Haemosporidia. Due to a lack of differentiation into cytoplasm and nucleus and the absence of a life cycle, some investigators are inclined to consider Anaplasma and Eperythrozoon as bacterial in nature, but this classification is rejected by Breed, et al. (6) due to unsuccessful attempts to grow the organisms in the absence of living cells. In addition, studies of the Anaplasma marginale body described by DeRobertis and Epstein (10) suggested multiple division of the parasite instead of the generally accepted binary fission characteristic of bacteria. Neitz (43) suggested that the multiplication of Eperythrozoon ovis may take place by budding since smaller forms were sometimes seen in contact with those of normal size. He noted one to three darker staining points in some of the ring forms which may be related to multiplication.

Foote, et al. (21) described the successful transmission of eperythrozoonosis in swine by using a filtrate of the infective agent. Experimental evidence is cited in which the organism was propagated in the yolk sac of chick embryos and passed through a twelve-pound filter. In addition, virus from an infected pig produced symptoms of the disease in a splenectomized calf, and the infective agent was described as

susceptible to neocarsphenamine therapy. A short time later, Foote (20) reported success in filtering the causative agent of anaplasmosis, using a three-pound Mandler filter. The use of the filtrate prolonged the incubation period in a splenectomized calf to 65 days as opposed to an average incubation period of 14 days. The author concluded that the infective agent was a virus and that the marginal Anaplasma body was probably an inclusion body.

The most accepted view on the classification of Anaplasma is to consider it within the group of intracellular parasites of protozoan nature. Eperythrozoon is classified in the same order as Anaplasma (6), but it is usually considered to be a Rickettsial organism (41). Lotze and Yiengst (38) describe the anaplasma as being segmented and containing a definite number of small sporoid bodies. The occurrence of extra-erythrocytic bodies is proposed, with the suggestion that this represents the infective form which develops into the anaplasma after entering the erythrocyte. Franklin and Redmond (24) observed projections or tails extending from typical Anaplasma bodies, and postulated that this may represent one stage in the normal development of Anaplasma. Espana, et al. (18) also observed tails or comet forms of the Anaplasma body by the use of phase contrast and electron microscopy on infected hemolyzed erythrocytes. These workers also ascribed the property of mobility to the organism and concluded that it was probably a true parasite belonging to the Protozoa. Ristic, et al. (55) demonstrated the existence of intercellular and submicroscopic bodies in addition to the classical marginal Anaplasma body by employing fluorescein conjugated antibody to locate antigenic structures. DeRobertis and Epstein (10) observed the mass of the parasite to be constituted of tightly packed elementary

bodies, and that in addition to the bodies present within the mass of the parasite, a large number of them were dispersed throughout the erythrocyte. Ristic (51) presented a detailed study of the Anaplasma body, and by means of electron microscopy described three distinct morphological forms associated with the development of Anaplasma marginale. The classical marginal inclusion body constituted the largest form and was made up of one to eight intermediate initial bodies. A smaller form or polyhedral body is described as a subunit of the initial body. Breed, et al. (6) classified Eperythrozoon and Anaplasma in the order Rickettsiales in which order the intracellular parasites of protozoa are provisionally assigned. Anaplasma is further classified in the family Anaplasmataceae, and Eperythrozoon is placed in the family Bartoncellaceae due to its extra-cellular location.

Incidence

Due to the mild nature of anaplasmosis and eperythrozoonosis in sheep, very little work has been done to determine their incidence. Neitz et al. (45) observed that fatal terminations due to eperythrozoonosis in sheep had not been noted, and prognosis under stable conditions was good. In a later study, Neitz (43) noted that even though mortality of eperythrozoonosis in sheep is low, the disease if widespread may be of considerable economic importance due to constitutional disturbance and rapid loss of condition. Quinlan, et al. (48) concluded that anaplasmosis runs a mild course in non-splenectomized sheep, but is more severe in splenectomized sheep with carrier infections. In previous work, these workers noted that many of the local sheep at

Onderstepoort, South Africa, were carriers of anaplasmosis although naturally occurring cases had never been observed. Splitter, et al. (61) noted that the generally mild nature of anaplasmosis in sheep presupposes that it may be more widespread than at first recognized, and that conditions of stress or increased individual susceptibility are required before the disease manifests itself in the clinically acute form. Gilbert (26) reported what appeared to be clinical cases of anaplasmosis following poor grazing seasons when sheep were in a debilitated condition. Ryff, et al. (56) detected anaplasmosis in a flock of sheep suffering from copper intoxication.

Boynton, et al. (5) devised a serum reaction test which was reported to detect animals recovering from anaplasmosis or recently recovered carrier-infected animals. Addition of two drops of serum to two cc. of distilled water followed by thorough shaking produced immediate cloudiness in positive cases. Simpson and Sanders (57) described three procedures which could be used for detection of carrier infections of anaplasmosis. These involved splenectomy of carrier-infected animals, the injection of carrier-infected blood into susceptible splenectomized animals, and the injection of carrier-infected blood into disease-free animals. Ristic, et al. (55) reported that the use of unlabeled immune serum on *Anaplasma* infected blood inhibits the fluorescent reaction and may be employed to test unknown serum for the presence of specific antibody using the fluorescent antibody technique.

The complement-fixation test is probably the most effective tool in detecting carrier-infected animals of anaplasmosis (25). Splitter, et al. (62) observed a cross antigenicity between *Anaplasma ovis* and *Anaplasma marginale* in the complement fixation test, the *Anaplasma*

marginale antigen giving equally accurate results when employed in the test for the presence of Anaplasma ovis infection. Gates, et al. (25) reported an average accuracy of 91.8% in the use of the complement-fixation test for diagnosing anaplasmosis. Twiehaus (65) has since reported an accuracy of 98%, emphasizing that standardization of the test is of primary importance in providing a highly accurate and efficient diagnostic tool in the field of anaplasmosis. Splitter (60), working with swine, demonstrated that the complement-fixation test may be useful in the laboratory diagnosis of acute eperythrozoonosis, but that it is of no value in determining the carrier-infection status of an animal. The use of Anaplasma antigen in the test for eperythrozoonosis was shown to give non-specific reactions, and the accuracy of the test was not as great as when an antigen prepared from Eperythrozoon was used.

Neitz (43) pointed out that post-mortem examination alone would hardly be of assistance in arriving at a diagnosis of eperythrozoonosis in sheep. Effective diagnosis of acutely affected animals is accomplished by observing typical organisms in stained smears of the peripheral blood.

Pathogenesis

Anaplasmosis. DeKock (8) noted that the only important alteration produced by anaplasmosis in non-splenectomized sheep was a slight oligocythemia. However, in splenectomized sheep infected with Anaplasma he reported the most characteristic changes were produced in the liver and consisted of extensive erythrophagocytosis and desquamation of Kupffer cells. DeKock and Quinlan (9) reported that sheep affected with

anaplasmosis had severe, often fatal, relapses 9 - 13 days after splenectomy. Typical symptoms included a febrile reaction, anemia and in some cases icterus. Splitter, et al. (62), in the first report on anaplasmosis in sheep in the United States, observed a pathogenesis similar to that described by earlier workers. A maximum erythrocytic infection of 19% was reached 26 days after experimental inoculation, and a progressive anemia was concurrent with the gradual reduction in the number of Anaplasma bodies. Erythrocyte counts reached a low of 3,000,000 cells per cubic millimeter of blood. The characteristic drop in erythrocytes which corresponded to a decrease in the number of Anaplasma bodies demonstrated in blood smears of infected animals stimulated theories concerning the role these bodies play in erythrocytic destruction. Lotze and Yiengst (38) inferred that this destruction was in some way brought about by the growth and multiple division of the anaplasms within the cell. Foote (20) contended that the Anaplasma body was probably an inclusion body and that the destruction of erythrocytes was due to the action of a virus. Ristic (52) reported increased osmotic fragility of erythrocytes in animals infected with Anaplasma marginale, but observed that there was no indication of intravascular hemolysis. The production of an autoantibody against the erythrocytes of infected animals was demonstrated, and supporting data suggested that the erythrocytes, sensitized with autoantibody, were removed from the circulation and broken down by the reticule-endothelial system.

According to Breed, et al. (6) Anaplasma ovis resembles Anaplasma marginale very closely, but differs by its position in the erythrocyte and its pathogenicity. Approximately 35% of the organisms are situated at or near the center of the erythrocyte and 65% are situated at or near

the margin of the cell. In Anaplasma marginale infections 90% of the organisms are situated at or near the margin of the erythrocyte. From one to two organisms are usually visible in an infected erythrocyte stained with Giemsa, and rarely three or four organisms may be seen in the same cell. During the height of reaction as many as 5% or more of the erythrocytes may be parasitized. Anaplasma ovis cannot be transmitted to cattle, and the Anaplasma marginale of cattle will not infect sheep, although sheep have been shown to retain the virulent organism from the bovine (6), (48), (49), (58), (61). In addition to being infective for sheep, Anaplasma ovis has been found to infect goats (6), (9), (61) and the Blesbuck antelope (6), (17). Post and Thomas (47) inoculated susceptible splenectomized sheep with blood obtained from elk responding positively to the complement-fixation test for anaplasmosis. Positive complement-fixation reactions were obtained from serum samples 15 days after inoculation and for 68 days, after which they were negative. Anaplasma bodies were not detected, and there was no change observed in the packed cell volume of the inoculated sheep. Challenge with known infective blood from donor sheep failed to produce clinical symptoms. Splenectomized calves used in this experiment failed to develop positive complement-fixation tests after inoculation with infected elk blood, and the calves developed acute anaplasmosis when challenged with known Anaplasma-infected blood of bovine origin.

Animals infected with anaplasmosis and eperythrozoonosis remain carriers of these diseases for variable periods of time and may be carriers for life (6). Rees and Underwood (50) reported finding one infected cell for every two-thousand non-infected cells in the

peripheral blood of cattle during the carrier stage of anaplasmosis. Studies by Ristic (51) in which the persistence of submicroscopic initial bodies in the erythrocytes of animals recovered from the clinical signs of anaplasmosis is demonstrated, represent a way in which carrier infection may be perpetuated.

Eperythrozoonosis. In what is probably the first description of eperythrozoonosis in sheep, Neitz, et al. (45) reported ring-shaped bodies 0.5 - 1.0 micron in diameter which appeared in blood smears 4-15 days after inoculation of susceptible sheep with the organism. The organisms were found lying supracellularity on the erythrocytes, but a greater number were found free between the cellular elements in the blood smears. The observed organisms appeared to be flat rings, ovoid, irregularly shaped triangles, rod, dumbbell and coma-shaped. Part of this variation was attributed to injury during the process of drawing the film when making a blood smear. The parasites made their first appearance about the time of a mild febrile reaction which was the first symptom. It appeared that the number of parasites suddenly decreased at the time anemia first appeared. The condition of the blood tended to become normal again after four weeks, but relapses were noted. Anemia was a constant and regular symptom and a slight icterus was observed in some cases. Red cell counts were reported to drop as low as 1,500,000 per cubic millimeter. Erythrophagocytosis was a typical feature produced by the disease. The onset of anemia was reported by Littlejohns (39) to occur 6-8 days after parasites were first observed in small numbers. Large pale erythrocytes similar to those observed in the macrocytic anemia of anaplasmosis were reported, and it was postulated that exogenous enzymes of the parasites may cause digestion of the

erythrocyte contents. Two splenectomized sheep were described in which an acute attack of eperythrozoonosis had subsided prior to splenectomy. In one animal the organisms appeared on the 13th day after splenectomy, increased to the 22nd day and were absent from the 24th to 38th day after splenectomy. In the second animal, Eperythrozoon ovis appeared on the 19th day and then were absent till the 38th day when they reappeared and increased till the 45th day. After further study on eperythrozoonosis in sheep, Neitz (43) noted that the organisms may be demonstrated in the peripheral blood of an infected animal for 6 - 42 days with an average period of 14 days. The recovery from infection with auto-sterilization was not observed, and it was concluded that an equilibrium between the parasite and host is developed. Irregular recurrence of the parasite in the blood stream after splenectomy was also reported.

The species of Eperythrozoon isolated from sheep in the United States by Jensen (31) appeared to be similar in every respect to the first description of Eperythrozoon ovis offered by Neitz, et al. (45). The host specificity of Eperythrozoon ovis was established by Neitz (44) and used as a basis for separating bovine and ovine strains by Jensen (31). Splitter (59) demonstrated the host specificity of bovine, ovine, and porcine strains of eperythrozoonosis. Enigk (17) reported the successful transmission of Eperythrozoon ovis to the Blesbuck antelope. Breed, et al. (6) indicated that goats and splenectomized calves may be susceptible to Eperythrozoon ovis.

An important feature of eperythrozoonosis is its ability to exist concurrent with and apparently aggravate other conditions. Jensen (31) reported that Eperythrozoon wenyoni tends to complicate anaplasmosis in cattle under experimental conditions. Foote, et al. (23) stated that

the etiological agents of eperythrozoonosis and anaplasmosis appear to compete for supremacy for several days before one or the other becomes dominant in splenectomized calves. Gledhill and Dick (27) reported that Eperythrozoon coccoides by itself is a harmless blood parasite of mice, but it has the ability to cause a severe or fatal hepatitis to develop when the normally avirulent mouse hepatitis virus is present. Gledhill and Niven (28) demonstrated that filtrates of Gram-negative bacteria which were practically non-toxic for normal mice were highly toxic to mice infected with Eperythrozoon coccoides. Lotze and Yiengst (37), during the course of investigations on bovine anaplasmosis, noted that eperythrozoon always seemed to be present during the anaplasma phase of experimental infection. As reported by Splitter, et al. (62), the first observation of anaplasmosis in sheep in the United States was associated with a simultaneous infection with Eperythrozoon ovis in all the cases studied.

Transmission

The inoculation of infected blood has been used in transmission studies for experimental work on anaplasmosis and eperythrozoonosis (17), (31), (43). In one instance, Foote et al. (21) reported the successful transfer of an agent thought to be the cause of eperythrozoonosis by the inoculation of tissue and blood filtrates. Berrier and Gouge (4) reported a case in which Eperythrozoon were present on the erythrocytes of a pig that died with anemia before one week of age. Enigk (16) indicated that intrauterine infections did not occur with Anaplasma, Theileria, and Babesia. Reported cases are explained on the assumption

that a local temporary connection between maternal and fetal blood was established through the rupture of small vessels in the placenta, or due to damage of the walls of blood vessels by toxins. Splitter (58) asserted that in-utero infection with anaplasmosis is possible, but it is rather insignificant and not an important means of perpetuating the disease. Jensen (31) reported that natural transmission of eperythrozoonosis had not been proved, but was suspected to be accomplished by arthropod vectors. Dikmans (12) presented a review of the various means of transmission of anaplasmosis by ticks, insects and other methods. It was concluded that insects act as mechanical transfer agents with no conservation or developmental stages of the etiological agent taking place within them. Twiehaus (65) listed ticks, horseflies, stableflies, mosquitoes, deerflies and hornflies as biological vectors, and these and other arthropods as mechanical vectors of anaplasmosis. Marsh (41) stated that the natural transmission of eperythrozoonosis in sheep is unknown.

Effects of Splenectomy

Experimental infection of sheep with Eperythrozoon ovis and Anaplasma ovis is greatly facilitated by the use of splenectomized animals. The function of the spleen in these and other diseases has been a subject for intensive study. The earliest report of splenectomy of large domestic animals, according to Davis (7), is that of Warthin in 1903. He operated on sheep and goats using chloroform anaesthesia, and 50% of the animals died of shock. DeKock and Quinlan (1926) were among the first to report the severe relapses occurring in sheep affected with

anaplasmosis after splenectomy (9). In order to determine the cause for the extensive changes produced in the liver of such sheep, DeKock (8) studied the effects of splenectomy on healthy sheep as well as on those infected with anaplasmosis, enzootic icterus, and bacterial icterus. Since no specific pathological anatomical changes were noted following splenectomy of the healthy sheep, it was concluded that the reticulo-endothelial system of the sheep can deal with normal blood destruction after splenectomy without manifesting specific lesions. Abnormal blood destruction in the splenectomized animal was manifested by characteristic changes in the liver. Rees (49) duplicated the early work of DeKock and Quinlan and concluded that the important changes produced in the blood of splenectomized carrier infected animals were produced by the infective agent and not by the surgical procedure.

Foote, et al. (22) in an attempt to determine the activity of the spleen as a defense mechanism against anaplasmosis, injected small amounts of infected blood directly into the spleen of three cows after ligating the splenic vessels. All three animals contracted acute forms of anaplasmosis and the authors concluded that the spleen was therefore not the first line of defense against the disease. Neitz (43) presented evidence that the spleen has a significant role in the mechanism of immunity to protozoan infections. Removal of the spleen from two known Eperythrozoon ovis carrier-infected animals resulted in a reappearance of the parasites in large numbers in the blood stream, and daily examination of blood smears showed irregular recurrence of Eperythrozoon ovis as long as 15 months after splenectomy. Unexpected results were obtained in an experiment by Ristic, et al. (54) in which cortisone treatment was used in an attempt to depress the protective forces of an Anaplasma

carrier-infected animal. Cortisone treatment for approximately four weeks prior to splenectomy resulted in protection of the treated animals against relapses following splenectomy. It was suggested that the cortisone might have stimulated extrasplenic tissue to sufficient anti-anaplasma activity to compensate for the removal of the spleen. Disappearance of complement-fixing antibody from the serums of splenectomized Anaplasma carrier-infected calves was encountered when cortisone treatment was not administered. The disappearance of antibody corresponded with Anaplasma invasion of the erythrocytes and subsequent relapse of the infected animal. Persistence of the antibody after splenectomy and protection against relapse were observed in animals treated with cortisone. It was concluded that the spleen has a distinct role in producing and maintaining Anaplasma complement-fixing antibody, but that in animals treated with cortisone other organs may function in setting up immunity.

Ristic and Sippel (53) proposed that the compensatory interaction between the spleen and other tissues capable of erythropoiesis is significant in developing and maintaining resistance to the effects of anaplasmosis. Removal of the spleen when the bone marrow is depressed due to Anaplasma infection, and when there is a lack of extramyeloid hematopoietic activity, disturbs the existing balance of defense forces and results in anemia. It is known that the spleen also participates in but is not essential for antibody production. Ortega and Mellors (46), using the fluorescent antibody technique, found that specific antibody produced in response to a variety of antigens, was formed in the germinal centers of lymphatic nodules and in the cytoplasm of mature and immature plasma cells. As proposed by Ristic and Sippel (53), the structure of

the spleen may be involved in the defense mechanism against blood infections. Since the spleen possesses blood sinuses in addition to ordinary capillaries, the rate of blood flow is decreased as it passes through the organ. It was proposed that infected erythrocytes are thus allowed maximum exposure time to the antibody-producing plasma cells and the potentially phagocytic cells which line the sinuses in the spleen.

Applications of Electrophoresis

Although the electrophoretic analysis of serum proteins can seldom be expected to yield a specific etiological diagnosis, the technique of electrophoresis is of considerable interest in the study of disease processes. Luetscher (40), in discussing the limitations and applications for electrophoretic studies, points out the flexibility and artificiality of any single set of conditions used in the electrophoretic analysis of blood proteins.

The simplest type of observations which can be made by electrophoresis is the enumeration of boundaries. Tiselius (64) described the electrophoretic fractions of plasma proteins by identifying albumin, fibrinogen, and the three globulin fractions which were named alpha, beta, and gamma in the order of their mobilities. The nomenclature of Tiselius for the well defined fractions noted in human plasma becomes inadequate when applied to the complex animal serums such as those of sheep in which as many as ten or more peaks may appear (11), (40).

Deutsch (11), in an electrophoretic survey of normal animal plasmas, concluded that the only component which can be evaluated with accuracy is the albumin. Hoffman (30) and Luetscher (40) indicated that

electrophoretic fractions, far from homogeneous in chemical composition and biological functions, may have the same mobility. Luetscher proposed that there may be qualitative changes in serum proteins that are altered by disease without any corresponding quantitative changes. He suggested that different buffer solutions may be employed in electrophoretic studies of animal serums which would give more satisfactory results than those now in general use.

Stauber (63) states that where serum changes have occurred as a result of parasitic infection the general picture most often seen is a reduction in albumin accompanied by an increase in one or more of the globulin fractions. Leland (35) reported similar findings, but stated that there was apparently no evidence to indicate that the marked increases observed in the globulin of infected animals were in response to the antigenic nature of the worm. An increase in globulin and reduction in albumin, according to Hoffman (30), is characteristic in most acute infections. Luetscher (40) stated that the common denominator of almost every pathological condition is a relative or absolute decrease in the serum albumin. This reduction may be a measure of the severity of the condition and occurs earlier than the increase in globulin. Luetscher also noted that the gamma-globulin fraction is not entirely active antibody, and some antibody has been found in other globulin fractions. Koprowski, et al. (33) confirmed evidence by other investigators showing that even though neutralizing antibody against certain viral diseases was located predominantly in the gamma-globulin fraction of immune serums, there was no significant difference between the electrophoretic patterns of virus-immune and normal serums.

The effect of splenectomy on serum proteins as reported by

Dimopoulos, et al. (13) was a transient decrease in total serum protein, gamma globulin, and total globulin based on grams per 100 ml of serum. These values returned to pre-splenectomy levels about one month after the operation. A transient increase in alpha globulin was noticed during the second week after surgery. However at no time did the values of serum proteins fluctuate beyond the normal limits established for the species of animal. Similar results were reported by Hoch-Ligeti (29) on the protein patterns of patients undergoing surgery, except in this report there was no decrease in total serum proteins. Luetscher (40) found that severe bodily injury was usually followed by an increase in alpha-globulin.

In another study involving splenectomized calves which were inoculated with Anaplasma marginale, Dimopoulos, et al. (15) noted a decrease in all serum proteins 5 days after infection. Approximately 15 days after infection, alpha and beta globulins began to rise followed by an increase in gamma globulin 26 days after infection. Total protein levels were at a low point as the number of Anaplasma bodies decreased. The drop in total proteins was attributed mainly to the albumin fraction. Dimopoulos, et al. (14) noted a severe decrease in globulins (especially the gamma fraction) concurrent with an established infection of eperythrozoonosis in calves. It was postulated that the hypoglobulinemia may be a significant method by which the disease manifests its pathogenesis due to a lowered defense mechanism.

The freezing of sheep blood serums at -20°C . was found by Kuttler, et al. (34) to cause no significant changes in the electrophoretic patterns. However, storage of the frozen serums longer than 8 months resulted in a small but significant increase in the beta globulin fraction.

Neely and Neill (42) stated that recovery of protein fractions in paper electrophoresis is only possible to within about \pm 5% under optimal conditions, and that small numerical variations must not be regarded as unduly significant when pathological serum is employed.

MATERIALS AND METHODS

Twenty-five adult sheep of mixed origin and various sources were selected for the experiment from a flock maintained by the University for experimental purposes. The sheep were sheared April 29th and identified with ear tags and corresponding back numbers applied with black paint.

Sheep No. 24 was splenectomized May 1st, following in general the technique described by Quinlan, et al. (48) except that local anesthesia consisting of the infiltration of 2% procaine was used, and 1/8 inch umbilical tape was used to ligate the splenic vessels and suture the skin. The remaining animals with the exception of Nos. 18, 22, and 23 were splenectomized May 2nd by members of the senior veterinary medical class in Surgical Exercises. Sheep Nos. 18, 22, and 23 were splenectomized May 3rd. The same procedure was followed in surgery on all sheep in the group with the exception of sheep No. 7 and No. 20 which had been previously splenectomized and were known to be carrier-infected with anaplasmosis. These two animals were included in the experiment as controls, and were submitted to surgery for an exploratory laparotomy as a measure of environmental uniformity. Surgery was conducted under field conditions using semi-sterile technique. Two cc. of

Combiotic* was administered intramuscularly to each sheep at the time of surgery as a prophylactic measure against the accidental introduction of infection. Sheep No. 8 suffered extensive loss of blood during surgery when the splenic vessels were accidentally ruptured prior to being ligated. Skin sutures were removed 10 days after the operations.

Blood samples were collected from each animal on May 1, May 5, May 8, May 10, May 12, May 15, May 17, May 19, May 22, May 24, May 26, May 29, May 31, and June 3. Clotting was inhibited by dipotassium ethylenediamine tetra acetate (EDTA).** In addition, serum samples were collected on May 5, May 12, May 19, May 29, and June 3, and stored at -20°C .

Packed cell volume was determined on each blood sample using a Wintrobe hematocrit tube and centrifuging the samples for 30 minutes at 3000 rpm.

Red blood cell counts on each animal in the experiment were made on May 8, May 10, May 12, May 17, May 19, May 24, May 26, May 31, and June 3. The counts were performed with a Coulter Counter*** using a dilution of 1:50,000 with triple filtered isotonic saline solution, the dilutions being prepared manually with specially designed pipettes. The Coulter Counter was operated at an aperture current (APC) setting of 6 and threshold of 8, and the actual count was obtained by correcting the figure obtained from the coulter counter with a reference table for that purpose.

* Haver-Lockhart Laboratories, Kansas City, Missouri. (400,000 units Penicillin G, Procaine and 0.5 Gm Dihydrostreptomycin as sulfate per cc.)

** Cambridge Chemical Products, Inc., Dearborn, Michigan.

*** Coulter Electronics, Hialeah, Florida.

Blood smears were prepared from each animal immediately following collection and fixed for 3 minutes in 95% ethyl alcohol. At the termination of the experiment, all the slides were stained at one time with Hem-O-Sen,* a "dip" type differential blood stain, and examined under a microscope for the presence of Anaplasma and Eperythrozoon bodies.

Complement-fixation tests were conducted on the serum samples using the procedure prepared and recommended by the committee on a standardized complement-fixation test for anaplasmosis (3).

Serum protein separations were performed on samples that were collected on May 5, May 12, May 19, May 29, and June 3, using a Spinco Model R Paper Electrophoresis System.** This system consists of two Durrum-type electrophoresis cells, a model RD-2 Duostat regulated power supply, and a model RB Analytrol densitometer and integrator. Spinco B-2 Buffer, Veronal, pH 8.6, package form (2.76 gm. diethyl barbituric acid, 15.40 gm. sodium diethyl barbiturate 0.075 ionic strength) and Schleicher and Schuell 2043-A mgl. paper strips 3 cm. wide by 30.6 cm. long were used in the cells; 0.006 ml of serum was applied to each strip by use of a micropipette and sample applicator. A current of 7½ milliamperes per cell (0.937 milliamperes per strip) was applied for 10 hours. The connector plugs to the cells were reversed 5 minutes before the strips were removed for the purpose of reversing the polarity within the cells. At this time the strips were dried in a pre-heated oven at 120-130 degrees C. for 30 minutes, and stained with bromphenol blue dye in alcoholic solution (Spinco Procedure B, technical bulletin No.

* Hoppers Laboratories, Houston, Texas.

** Spinco Division, Beckman Instruments, Inc., Belmont, California.

TB 6050A). Each strip was scanned photometrically on the Analytrol recording scanner and integrator equipped with a B-5 cam and 550 millimicron interference filters using a 1.5 mm. slit width. Relative concentrations of the protein fractions were determined by use of the "lowest point between peaks" method of delimiting components on the Analytrol trace.

In order to convert these relative concentrations of serum fractions to absolute values (Gm./100 ml. of serum), total protein determinations were made on all serum samples using the "Improved" Biuret method for total protein by Ferro and Ham (19). Optical density was read using a Coleman Junior Spectrophotometer* adjusted for 100% transmittance at a wavelength of 540 millimicrons.

EXPERIMENTAL PROCEDURES AND RESULTS

The packed cell volume (PCV) of all animals decreased as a result of surgery, but showed a partial recovery six days after splenectomy in most instances. Approximately half of the sheep exhibited anorexia, depression, and temperatures of 104° - 105° F. seven to ten days after surgery. Sheep No. 1 died of peritonitis on May 9th.

It was not possible to demonstrate *Anaplasma* or *Eperythrozoon* organisms in stained blood smears prior to splenectomy. The first evidence of *Anaplasma ovis* appeared in blood smears ten days after splenectomy. *Eperythrozoon ovis* was usually first observed 15 - 18 days after splenectomy. The greatest number of *Eperythrozoon* organisms

* Coleman Instruments, Inc., Maywood, Illinois.

was noted about 21 days after splenectomy.

Eperythrozoon ovis appeared in blood smears of all animals in the group, fourteen sheep showing numerous organisms and ten sheep exhibiting a slight to moderate infection. Nine sheep reacted positively to the complement-fixation test for anaplasmosis, using a 1:5 dilution of serum. Anaplasma bodies were demonstrated in blood smears of seven of these animals. The other two sheep (No. 7 and No. 20) which reacted positively to the complement-fixation test had been splenectomized sometime previous to the beginning of this study, and were carrier-infected with Anaplasma ovis.

The occurrence of Anaplasma ovis in positive blood smears was generally quite limited, with a predominance of Eperythrozoon ovis present (Plate I, fig. 1).

On the basis of hematocrit determinations and red blood cell counts, only two sheep in the group exhibited acute symptoms. Sheep No. 11 developed a severe anemia with a PCV of 17% and a red blood cell count below 5,000,000 cells per cubic millimeter. Increased fragility of the erythrocytes was evident in blood smears and in hemolyzed serum following centrifugation for hematocrit determinations. This animal died June 18th (47 days after splenectomy) with lesions of acute pneumonia in addition to the anemia. Plate I, fig. 2, is a photomicrograph of a blood smear prepared from sheep No. 11 twenty-two days after splenectomy.

Sheep No. 18 developed an acute dual infection with Anaplasma ovis and Eperythrozoon ovis. The PCV dropped to 13% with hemolysis and icterus noted in the serum. Red blood cell counts fell to a low of 2,770,000 cells per cubic millimeter. The acute symptoms reached a peak thirty-one days after splenectomy with a gradual recovery taking

place thereafter.

Symptoms of the other sheep in the group were relatively mild with hematocrit levels generally of 30% or above and red blood cell counts ranging from 7,000,000 to 12,000,000 cells per cubic millimeter. Anisocytosis was observed in the blood smears of most advanced cases of infection (Plate I, fig. 1, and Plate II, fig. 4). Erythrocyte fragility appeared most evident in blood smears where organisms were highly concentrated, usually between 20 and 24 days after splenectomy. The Eperythrozoon and Anaplasma organisms were generally absent in blood smears from two to four days following their first appearance, and then they reappeared in varying concentrations until termination of the observations 38 days after splenectomy. The appearance of numerous Eperythrozoon organisms in a blood smear was usually followed in several days by smears in which the number of organisms was reduced, sometimes to the point of being difficult to demonstrate.

It was not uncommon to observe stained blood smears in which the Eperythrozoon organism appeared as a small ring on the border of the erythrocytes (Plate II, fig. 3). These are typical of the signet ring and ring-shaped forms first described by Neitz, et al. (45).

The PCV levels of the seven sheep exhibiting an active dual infection with Anaplasma ovis and Eperythrozoon ovis and of the one sheep showing acute symptoms with Eperythrozoon ovis infection are included in Table 1.

In order to verify the accuracy of the complement-fixation test in detecting Anaplasmosis in carrier infected sheep, and to demonstrate the symptoms of anaplasmosis in sheep when induced separately from eperythrozoonosis, the following study was conducted.

Table 1. Hematocrit levels of sheep exhibiting active infections with Anaplasmosis and Eperythrozoonosis.

Sheep No.	Date													
	May 1	5*	8	10	12	15	17	19	22	24	26	29	June 1	3
4	38	35	35	34	32	34	30	34	33	33	31	29	30	29
6	44	28	26	28	28	30	30	32	31	32	30	29	33	35
10	39	27	29	25	28	26	26	27	28	26	26	27	27	28
11**	38	28	29	28	23	24	24	24	25	20	19	18	17	17
18	43	40	37	38	34	41	35	36	34	30	26	21	13	13
22	35	29	30	28	28	29	28	36	29	26	26	27	28	27
23	44	34	38	33	32	33	32	35	33	31	29	31	28	29
25	43	33	33	28	28	30	33	36	32	32	30	30	34	31

* First sample after splenectomy.

** Infected with Eperythrozoonosis only.

On September 21st, nearly five months after the sheep underwent surgery for splenectomy, 1 cc. of blood from sheep No. 18 (previously shown to have an active infection of Anaplasma ovis and Eperythrozoon ovis) was injected intravenously into sheep Nos. 21 and 24 (previously shown to have an active infection of Eperythrozoon ovis only) and No. 20 (previously shown to have a carrier-infection with Anaplasmosis). Blood samples were collected from the recipients prior to injection, and at regular intervals for 33 days after injection. The course of anaplasmosis infection in these three animals may be followed by observing their hematocrit levels in Table 2.

Eperythrozoon ovis was present in blood smears from sheep Nos. 21 and 24 prior to injection, and was observed in varying concentrations in subsequent blood smears from all three animals. Anaplasma bodies first appeared in the blood smears of sheep Nos. 21 and 24 on October 3rd (12 days after injection) and increased in number until October 12-14.

Table 2. Hematocrit levels of three sheep injected September 21st with blood from sheep No. 18.

Sheep No.	Date														
	:Sep.					:Oct.									
	21	26	28	30	3	5	8	10	12	14	17	19	21	23	24
20	35	37	34	34	32	34	32	35	31	31	32	36	32	34	36
21	28	33	30	31	31	30	29	31	29	28	27	21	10	7	died
24	29	32	31	29	32	32	31	33	29	30	23	16	10	11	14

Approximately 50% of the cells appeared to be parasitized with from one to three Anaplasma bodies in the samples taken after October 14th (Plate III).

The relative unimportance of the associated Eperythrozoon infection in the donor sheep was demonstrated by injecting 1 cc. of blood from sheep No. 8 into sheep No. 13 (both known to have been infected with Eperythrozoon but negative to the complement-fixation test for anaplasmosis). Hematocrit levels for the thirty days following injection varied between 31% and 36%. On October 31st, blood from sheep No. 24 (infected with blood from sheep No. 18 on September 21st) was injected into sheep No. 13, and the following hematocrit levels were recorded: October 31 - 33%; November 9 - 38%; November 16 - 35%; November 23 - 10%; death occurred on November 27.

These transmission studies indicate that the Anaplasma organism associated with Eperythrozoon ovis produced an acute infection when introduced into sheep which were refractive to the Eperythrozoon organisms. The fact that there were no mortalities in the initial studies when Eperythrozoon and Anaplasma infections occurred simultaneously is paradoxical by 67% mortality when Anaplasma ovis was introduced

into three Eperythrozoon refractive sheep.

Information relative to the effect of Anaplasma ovis and Eperythrozoon ovis upon the serum proteins of sheep was aspired during the course of this study to determine whether an immune response could be detected. The simplest means of evaluating serum protein is through the application of a paper electrophoresis system. Such a system is designed to separate the components of a solution which possess different electrophoretic mobilities, and to evaluate the amounts of such components. Serum samples which had been collected from the splenectomized sheep three days after surgery and thereafter approximately once a week for 30 days were used in this part of the study. In addition, serum from a non-splenectomized adult sheep in the same flock from which the splenectomized sheep originated was analyzed as a reference standard.

Separation of albumin and alpha, beta, and gamma globulins was achieved with little difficulty on serum samples collected from the non-splenectomized sheep and from the splenectomized sheep during the first 14 days following splenectomy. Serum samples collected later in the course of the infection demonstrated an increased homogeneity of the globulin fractions, making accurate classification in many cases confusingly arbitrary. The presence of four or more peaks in the globulin fraction added to the complexity.

In an effort to achieve greater definition of the protein fractions, seven different variations of time and current adjustment were employed with the electrophoresis system. The combination which appeared to give the best results was then used for all serum samples, although it was necessary in some cases to compare the results of several variations to arrive at a justified conclusion.

Nineteen of the twenty-four sheep studied showed an absolute increase in gamma globulin of greater than 10%. In seven animals the maximum response was noted in serum samples taken 17 days after splenectomy, in three animals the response was maximum at 27 days after splenectomy, and in the remaining nine animals maximum response appeared in the last sample taken which was 33 days after splenectomy. The increase in the gamma globulin fraction was generally the result of a decrease in another globulin fraction, since the total globulin component showed an increase of over 10% in only five instances.

Table 3 is a tabulation of those animals which showed an absolute increase in gamma-globulin as well as those animals which demonstrated an absolute increase in total globulins.

It is impossible to generalize the results of this analysis, but several trends can be noted. The beta-globulin fraction consistently declined between 10 and 17 days after splenectomy. The alpha-globulin declined in about 2/3 of the samples analyzed. Although there was variation between animals, the increase in gamma-globulin generally followed a decrease in beta-globulin. The A/G ratio tended to increase in serum samples subsequent to the first sample following splenectomy; sheep No. 11 is an exception, showing an increased globulin to albumin ratio which is typical of acute infections.

The electrophoretic patterns of serum fractions in terms of grams per 100 ml. of serum are presented in Table 4. Results of electrophoretic analysis of serum from a non-splenectomized sheep as well as normal values compiled by Altman (2) are presented in Table 5.

Table 3. Electrophoretic analysis of serums from 21 sheep which showed increases in the gamma-globulin fraction.

Sheep No.	% Increase : gamma-glob.	% Increase : total glob.	Albumin/Globulin ratio					Type of infection
			5 May	12 May	19 May	29 May	3 June	
2	38	-	.59	.65	.73	.89	.60	Eperythroozoon
3	23	-	.59	.51	.55	.74	.98	"
5	52	15	.49	.35	.43	.53	.44	"
6	20	3	.42	.47	.61	.71	.60	Dual*
7	20	-	.64	.72	.98	1.24	.73	Carrier**
8	46	10	.53	.64	.53	.47	.78	Eperythroozoon
9	18	-	.68	.61	1.02	1.01	.87	"
10	57	9	.51	.44	.56	.60	.56	Dual
11	50	29	.45	.39	.25	.24	.24	Eperythroozoon
12	62	-	.54	.46	.62	.98	.69	"
13	10	-	.66	.90	1.30	1.78	.97	"
14	45	-	.53	.44	.83	.85	.76	"
15	16	-	.57	.93	.87	.88	.68	"
16	5	-	.56	.70	.93	.98	.80	"
17	68	1	.48	.69	.62	.61	.89	"
18	62	-	.65	.74	1.01	.76	.59	Dual
20	14	37	.73	.47	.66	.68	.42	Carrier
21	23	-	.69	.86	1.18	1.32	1.05	Eperythroozoon
22	45	12	.53	.53	.41	.52	.53	Dual
23	53	-	.65	.86	.98	1.28	.70	"
25	48	19	.49	.43	.53	.78	.71	"

* Simultaneous infection with eperythroozonosis and anaplasmosis.

** Sheep reacted positively to anaplasmosis complement-fixation test, but Anaplasma organisms were not demonstrated in stained blood smears.

Table 4. Electrophoretic analysis of serum proteins (gms./100 ml. serum) in 24 sheep following splenectomy.

No.:	5 May				12 May				19 May				29 May				3 June			
	Alb.:	Alpha:	Beta:	Gamma:	Alb.:	Alpha:	Beta:	Gamma:	Alb.:	Alpha:	Beta:	Gamma:	Alb.:	Alpha:	Beta:	Gamma:	Alb.:	Alpha:	Beta:	Gamma:
2	2.62	1.67	1.02	1.72	2.94	1.61	0.89	2.05	3.06	1.26	0.56	2.38	3.41	1.20	0.39	2.24	2.39	1.10	1.02	1.88
3	2.95	1.54	1.45	1.98	--	--	--	--	2.65	1.75	0.79	2.29	3.08	1.27	0.46	2.45	3.27	0.92	0.45	1.96
4	2.80	1.69	1.16	2.06	--	--	--	--	3.66	1.18	0.39	1.79	3.45	1.02	0.41	1.73	2.91	1.36	0.36	2.20
5	2.46	1.25	1.04	2.73	2.11	1.56	0.97	3.50	2.52	1.20	0.44	4.15	2.90	1.11	0.53	3.66	2.29	1.17	0.54	3.46
6	1.90	1.28	1.02	2.21	2.41	1.49	1.09	2.49	2.81	1.30	0.68	2.66	2.92	0.99	0.63	2.48	2.55	1.26	0.43	2.59
7	2.92	1.28	1.11	2.14	3.22	1.59	0.53	2.36	3.90	1.21	0.46	2.33	4.24	1.07	0.40	1.95	3.06	1.17	0.45	2.56
8	2.28	1.49	1.69	1.14	2.67	1.83	0.40	1.91	2.51	1.31	0.64	2.80	2.11	1.36	0.37	2.77	2.88	1.13	0.50	2.09
9	2.67	1.54	0.81	1.58	2.57	1.64	0.68	1.94	3.33	1.10	0.34	1.82	2.99	0.88	0.44	1.65	2.86	0.89	0.54	1.86
10	2.31	1.50	1.18	1.83	2.15	1.75	0.94	2.22	2.74	1.49	0.57	2.88	2.15	1.10	0.58	1.92	2.30	1.24	0.45	2.38
11	2.40	1.93	1.39	1.99	2.23	2.03	0.42	3.23	1.73	1.49	0.79	4.57	1.49	1.11	0.50	4.56	1.60	0.87	0.91	4.98
12	2.79	1.79	1.51	1.83	2.34	1.29	1.56	2.30	3.18	1.62	1.40	2.09	3.48	0.92	0.51	2.12	2.96	0.89	0.45	2.96
13	2.63	1.70	0.82	1.45	3.23	1.49	0.66	1.45	3.97	1.24	0.42	1.39	4.36	1.06	0.26	1.14	3.36	1.45	0.41	1.60
14	2.44	2.02	0.98	1.61	2.09	1.59	1.04	2.09	3.38	1.59	0.42	2.06	3.42	1.21	0.48	2.33	2.94	1.66	0.34	1.88
15	2.64	1.22	0.96	2.43	3.50	1.44	0.50	1.82	3.17	1.15	0.38	2.10	3.28	1.05	0.40	2.29	3.10	1.32	0.46	2.82
16	2.43	1.39	0.80	2.19	2.99	1.32	0.78	2.16	3.69	1.23	0.41	2.31	2.74	0.85	0.37	1.57	2.84	1.05	0.37	2.13
17	2.20	1.60	1.44	1.57	2.78	1.06	0.83	2.44	2.79	1.18	0.98	2.32	2.81	0.98	1.02	2.64	--	--	--	--
18	2.60	1.26	0.81	1.94	2.72	1.24	0.40	2.03	3.21	0.84	0.35	1.99	2.86	0.91	0.38	2.45	3.02	1.25	0.74	3.12
19	2.53	1.42	1.34	1.98	3.01	1.18	0.53	2.10	4.04	0.90	0.45	1.65	2.89	0.69	0.36	1.60	3.25	0.80	0.62	1.92
20	2.98	1.20	1.01	1.84	--	--	--	--	3.31	1.20	0.69	3.10	3.43	1.41	0.59	3.09	2.35	1.05	0.58	3.94
21	2.69	1.68	0.76	1.47	3.25	1.65	0.50	1.65	4.03	1.30	0.46	1.65	3.88	1.14	0.31	1.49	3.72	1.39	0.34	1.81
22	2.20	1.47	0.87	1.84	2.29	1.59	0.66	2.06	1.92	1.41	0.61	2.66	2.20	1.12	0.62	2.44	2.14	1.04	0.50	2.48
23	2.61	1.38	1.12	1.49	3.04	1.21	0.63	1.72	3.36	1.04	0.42	2.00	3.83	0.84	0.31	1.83	2.63	1.13	0.35	2.28
24	2.38	1.87	0.91	2.10	3.03	1.40	0.61	1.76	3.45	1.13	0.39	1.64	3.56	1.08	0.42	1.76	3.12	1.46	0.47	1.77
25	2.17	1.70	0.83	1.91	2.04	1.57	1.10	2.11	2.81	1.63	0.82	2.83	3.46	1.06	0.65	2.71	2.82	1.25	0.65	2.10

Table 5. Electrophoretic analysis of serum protein in normal adult sheep. (Absolute values based on a total protein level of 6 gms./100 ml.)

Protein fraction	: Non-splenectomized adult : Normal values according		: sheep from University : to Koenig as compiled by	
	: flock		: Altman (2)	
	<u>Relative</u>	<u>Absolute (Gms.)</u>	<u>Relative</u>	<u>Absolute (Gms.)</u>
Albumin	41.1%	2.47	39%	2.34
Alpha-globulin	25.3%	1.52	17%	1.02
Beta-globulin	6.8%	.41	9%	.54
Gamma-globulin	26.7%	1.60	36%	2.16
A/G ratio		.70		.64

EXPLANATION OF PLATE I

- Fig. 1. Blood smear from sheep No. 18 (X 1250) twenty-two days after splenectomy. Anaplasma ovis and Eperythrozoon ovis are both evident with a greater number of Eperythrozoon organisms. Note anisocytosis.
- Fig. 2. Blood smear from sheep No. 11 (X 1250) twenty-two days after splenectomy. Eperythrozoon organisms are small, basophilic bodies on surface and edge of erythrocytes.

PLATE I

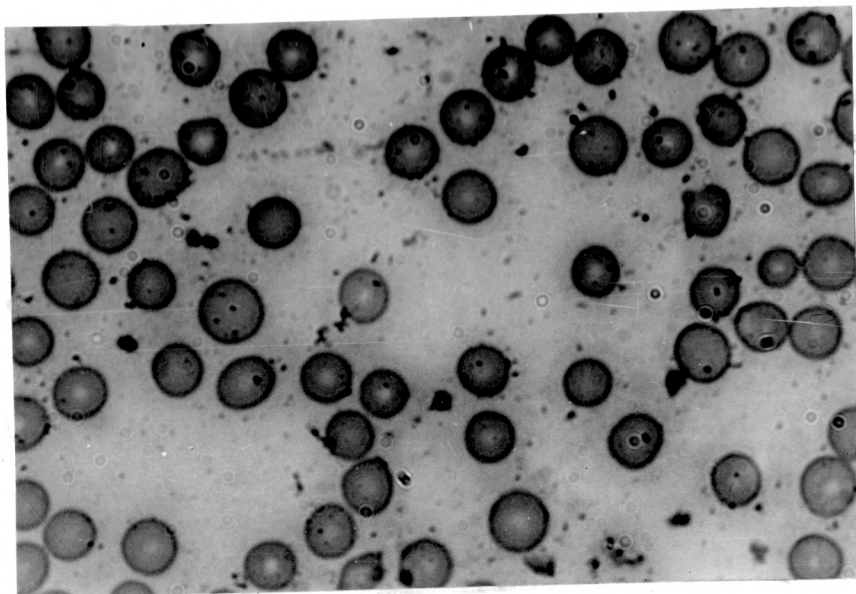


Fig. 1

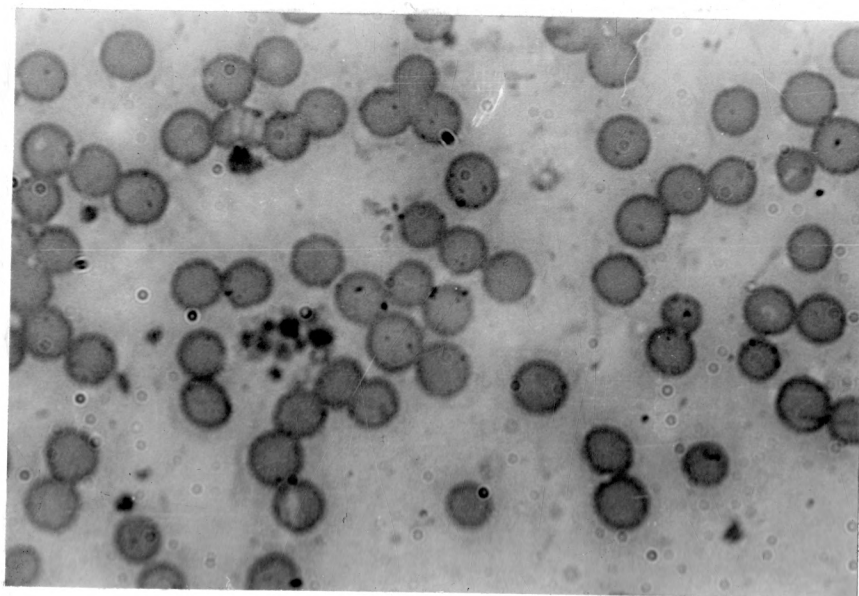


Fig. 2

EXPLANATION OF PLATE II

- Fig. 3. Blood smear from sheep No. 21 (X 1250) nearly five months after splenectomy showing persistence of Eperythrozoon ovis. Some of the organisms appear as small rings on the edge of the erythrocytes.
- Fig. 4. Blood smear from same animal as above (X 1250) thirty days after inoculation with positive Anaplasma ovis infected blood from sheep No. 18. Note anisocytosis.

PLATE II

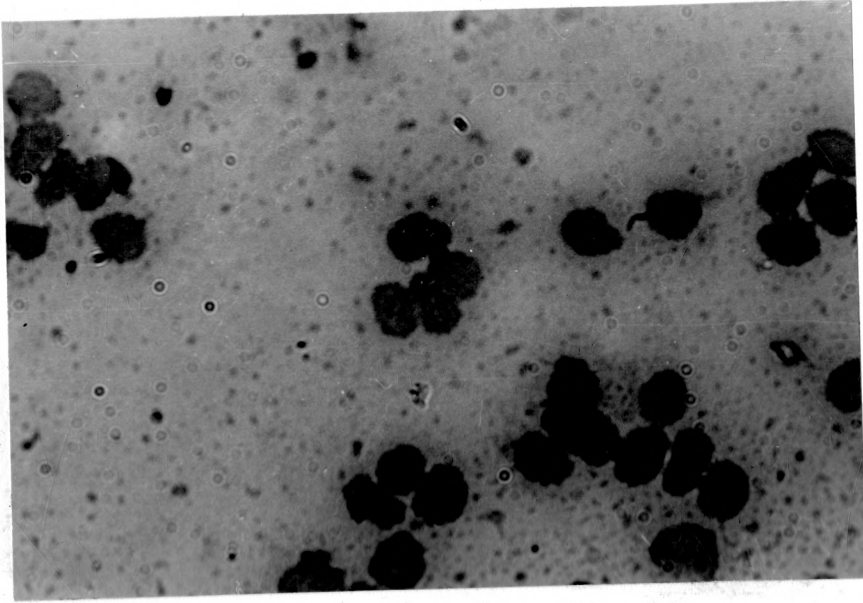


Fig. 3

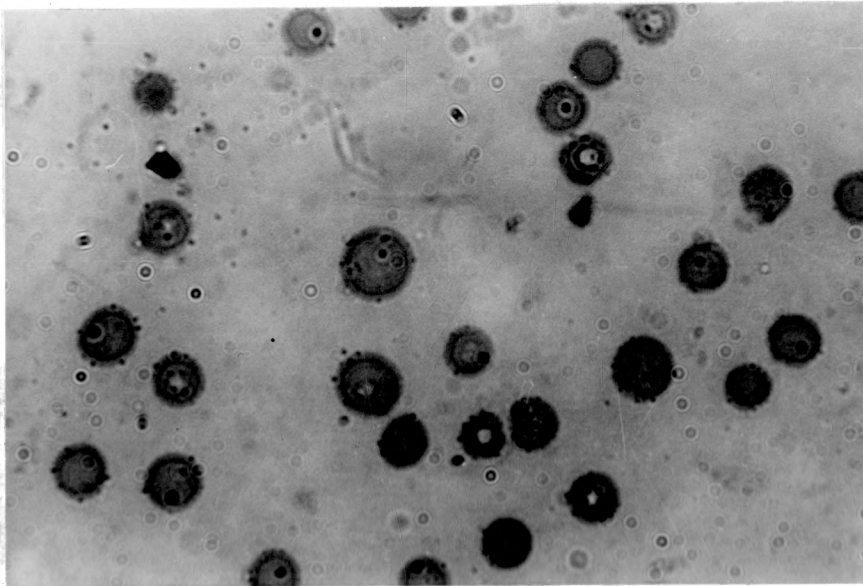
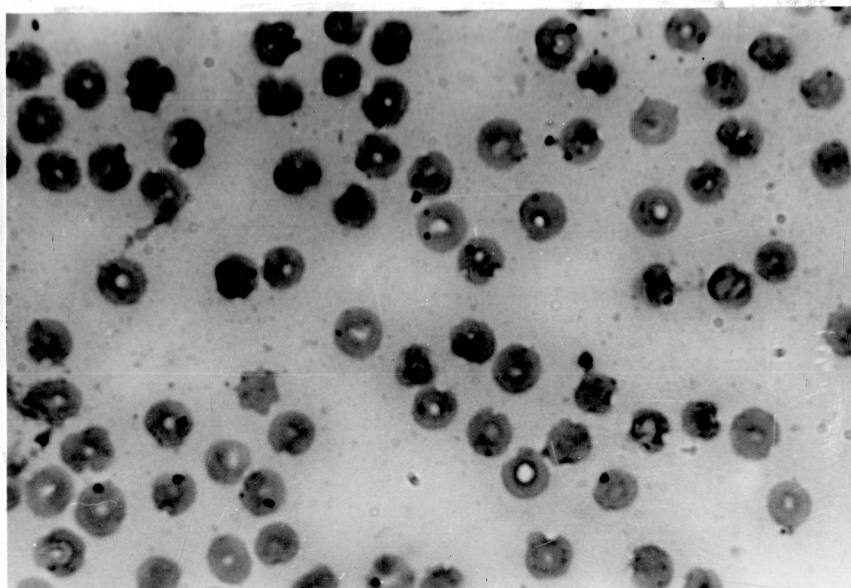


Fig. 4

EXPLANATION OF PLATE III

Stained blood smear of sheep No. 24 twenty-eight days
after inoculation with positive Anaplasma ovis infected
blood (X 1250).

PLATE III



DISCUSSION

The drop in packed cell volume following splenectomy was attributed to the effects of surgery rather than to the loss of the spleen, since it occurred in both animals which had previously been splenectomized. The depression noted one week after surgery was also attributed to the surgery, rather than to the incubating blood parasites, because it did not manifest itself in the entire group.

Eperythrozoon ovis infection appears to be a usually mild condition with a high incidence. Although it could not be demonstrated prior to splenectomy in blood smears from the twenty-five sheep included in this study, the organism was demonstrated in the twenty-four surviving animals within three weeks following removal of the spleen. That the spleen is significant in suppressing the appearance of Eperythrozoon ovis in the peripheral blood of carrier-infected animals was confirmed by demonstrating the organisms in blood smears from several of these sheep seven months after splenectomy. Neitz (43) reported the irregular occurrence of Eperythrozoon ovis in blood smears from sheep as long as 15 months after splenectomy.

The demonstration of Eperythrozoon ovis in the blood of sheep No. 7 and No. 20 which had been splenectomized at some time prior to this experiment may be attributed to several possibilities. These sheep may not have been carrier-infected with Eperythrozoon ovis previous to splenectomy, in which case the organisms were present due to exposure and infection subsequent to the time they were splenectomized. The other possibility is that the two animals relapsed with a dual infection of anaplasmosis and eperythrozoonosis following splenectomy, and the

Eperythrozoon organisms were present by virtue of their ability to recur for long periods after splenectomy. The latter explanation appears to be the most reasonable in view of the fact that the seven sheep which relapsed with anaplasmosis in this study all exhibited a dual infection with Eperythrozoon ovis.

The usually mild nature of Eperythrozoon ovis is illustrated by the fact that, with one exception, acute symptoms were not observed even in splenectomized sheep.

When Anaplasma ovis occurred in association with Eperythrozoon ovis, the battle for supremacy between the two organisms seemed to suppress the severe effects noted when Anaplasma ovis was introduced into splenectomized animals in which a latent infection with Eperythrozoon ovis existed. Apparently the Anaplasma organisms are somewhat attenuated by the antagonistic effects of Eperythrozoon ovis in simultaneous infections. Further evidence supporting this view was the observation of many Anaplasma bodies in blood smears from sheep immune to the effects of Eperythrozoon when infected with Anaplasma; a limited number of Anaplasma bodies were observed in the case of a simultaneous dual infection (see Plate I, fig. 1, and Plate III).

The use of electrophoretic analysis of serum proteins from sheep infected with Anaplasma ovis and Eperythrozoon ovis in a study of this nature is limited by the fact that the effects of surgical procedures on the animals cannot be separated from the effects due to infection. Dimopoulos, et al. (13) noted that it required about one month for the serum proteins of calves to return to normal following splenectomy. Therefore, when a carrier-infected animal is splenectomized, it is logical to assume that the effects of splenectomy and infection are

manifested concurrently. However, Dimopoulos also found that the values of serum proteins did not fluctuate beyond normal limits, indicating that any great change may be attributed to factors other than the splenectomy operation. Changes brought about by diseases of the blood cells may be difficult to evaluate due to secondary effects of the disease on the liver and hematopoietic system as well as the effects produced by fever and anemia.

In this study, where separation of the globulin fractions was at times difficult, the use of different buffer solutions as suggested by Luetscher (40) may have yielded more precise results.

The fact that the serum electrophoretic pattern revealed evidence of an acute infection in only one animal in this study is thought to be significant in characterizing anaplasmosis and eperythrozoonosis in sheep.

SUMMARY

Twenty-three sheep were splenectomized in an effort to detect latent infections of anaplasmosis and eperythrozoonosis. Two other sheep which had been previously splenectomized and were known to be carrier-infected with Anaplasma ovis were submitted to surgery and included in the experiment for comparison purposes. One sheep died from peritonitis seven days after surgery.

Packed cell volume and red blood cell determinations were recorded, and peripheral blood smears were prepared at regular intervals for 38 days following surgery. In addition, serum samples were collected and frozen for later use in the complement-fixation test and for serum

protein determinations.

Eperythrozoon ovis was demonstrated in blood smears from the twenty-four sheep which survived. The organism was first observed 15 to 18 days following surgery.

Nine sheep reacted positively to the complement-fixation test for anaplasmosis, including two sheep which had been splenectomized prior to the beginning of the experiment. Anaplasma ovis could not be demonstrated in blood smears from the latter two animals, confirming that they were carrier-infected, but was noted to first appear 10 days after splenectomy in the other seven sheep.

Acute symptoms, on the basis of hematocrit and red blood cell determinations, occurred in one animal exhibiting infection with Eperythrozoon ovis and in one animal which relapsed with a dual infection with Eperythrozoon ovis and Anaplasma ovis. Symptoms in the other twenty-two sheep were relatively mild.

Increased severity of symptoms were noted when splenectomized sheep refractive to Eperythrozoon ovis infection were infected with Anaplasma ovis. Death occurred in two of three sheep so infected, whereas no mortality occurred in seven animals in which a simultaneous infection of Eperythrozoon ovis and Anaplasma ovis was demonstrated.

Electrophoretic studies were conducted on the sheep serums collected at weekly intervals during the experiment. Analysis revealed a pattern typical of acute infection in only one animal. Gamma-globulin showed an increase of more than 10% in nineteen of the twenty-four sheep studied, but a total globulin increase of greater than 10% occurred in only five animals. The occurrence of more than three peaks in the serum globulin fraction and electrophoretic homogeneity in the latter part of

the study made the separation of distinct globulin fractions at times somewhat arbitrary.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. H. D. Anthony, Department of Pathology, for his suggestions and counsel concerning this study, and for his aid in the interpretation of microscopic findings.

Grateful acknowledgment to Dr. M. J. Twiehaus, Head of the Department of Pathology, for his guidance in the preparation of the thesis, and to Dr. E. H. Coles, Department of Pathology, for his aid and the use of laboratory facilities.

Special thanks to Dr. R. C. Piper for preparing the photomicrographs included in the thesis.

BIBLIOGRAPHY

1. Adler, S., and Ellenbogen, V.
A note on two new blood parasites of cattle, Eperythrozoon and Bartonella. Jour. Compar. Path. and Therap. 47(3):219-221.
2. Altman, P. L.
Blood and other body fluids, Federation of American Societies for Experimental Biology, 1961.
3. Anthony, H. D., Brock, W. E., Cross, R. F., Mott, L. O., Osebold, J., Poelma, L. J., Roby, T. O., Saulmon, E. E., Tunnicliff, E. A., and Twiehaus, M. J.
A standardized complement-fixation test for Anaplasmosis. Mimeo. USDA-ARS.
4. Berrier, H. H. Jr., and Gouge, R. E.
Eperythrozoonosis transmitted in utero from carrier sows to their pigs. Jour. Amer. Vet. Med. Assoc. 124(923):98-100. 1954.
5. Boynton, H. W., and Woods, G. M.
A serum reaction observed in Anaplasmosis. Preliminary report. Jour. Amer. Vet. Med. Assoc. 40(1):59-62. 1935.
6. Breed, R. S., Murray, E. G. D., and Smith, N. R.
Bergey's manual of determinative bacteriology. 7th ed. Baltimore: Williams and Wilkins, 1957. pp. 979, 983-984.
7. Davis, R. H.
Splenectomy of the calf for detection of Anaplasmosis. Southwest. Vet. 5(3):257-266. 1952.
8. DeKock, G.
A study of the reticulo-endothelial system of the sheep. Union So. Africa Dept. Agric. Rept. 13/14(1):647-742. 1928. Original not seen. Abstract in Biol. Abs. 5:2350. 1931.
9. DeKock, G., and Quinlan, J.
Splenectomy in domesticated animals and its sequelae, with special references to Anaplasmosis in sheep. Union So. Africa Dept. Agric. Rept. Div. Vet. Educ. and Res. 11/12(1):369-480. 1926. Original not seen. Abstract in Biol. Abs. 2:1689. 1928.
10. DeRobertis, E., and Epstein, B.
Electron microscope study of Anaplasmosis in bovine red blood cells. Soc. Expt. Biol. and Med. Proc. 77(2):254-258. 1951.

11. Deutsch, H. F., and Goodloe, M. B.
An electrophoretic survey of various animal plasmas. Jour. Biol. Chem. 161(1):1-20. 1945.
12. Dikmans, G.
The transmission of Anaplasmosis. Amer. Jour. Vet. Res. 11(38):5-16. 1950.
13. Dimopoulos, G. T., Foote, L. E., and Schrader, G. T.
Electrophoretic studies of bovine serum. I. Changes in the serum proteins after splenectomy. Amer. Jour. Vet. Res. 20(75):270-272. 1959.
14. Dimopoulos, G. T., Schrader, G. T., and Foote, L. E.
Electrophoretic studies of bovine serum. II. Concurrent hypoglobulinemia and natural infections of eperythrozoonosis. Soc. Exptl. Biol. and Med. Proc. 100(1):55-58. 1959.
15. Dimopoulos, G. T., Schrader, G. T., and Foote, L. E.
Electrophoretic studies of bovine serum. III. Serum protein changes in Anaplasmosis. Amer. Jour. Vet. Res. 21(81):222-225. 1960.
16. Enigk, K.
Die intrauterine ubertragung von blutprotozoen bei den haustieren. Deutsch. Tropenmed. Zeitschr. 46(6):153-160. 1942. Original not seen. Abstract in Biol. Abs. 17:1788. 1943.
17. Enigk, K.
Die empfänglichkeit der elenantilope fur Anaplasma ovis und Eperythrozoon ovis. Deutsch. Tropenmed. Zeitschr. 46(2):48-52. 1942. Original not seen. Abstract in Biol. Abs. 17:1785. 1943.
18. Espana, C., Espana, E., and Gonzalez, D.
Anaplasma marginale. I. Studies with phase contrast and electron microscopy. Amer. Jour. Vet. Res. 20(78):795-805. 1959.
19. Ferro, P. V., and Ham, A. B.
Improved Biuret method for total protein. Lab-Trol Bulletin 101. Dade Reagents, Inc., Miami, Fla. 1955. 4 pp.
20. Foote, L. E.
New information on Anaplasmosis. No. Amer. Vet. 35(1):19-21. 1954.
21. Foote, L. E., Brock, W. E., and Gallaher, B.
Ictero-anemia, Eperythrozoonosis, or Anaplasmosis-like disease of swine proved to be caused by a filtrable virus. No. Amer. Vet. 32(1):17-23. 1951.

22. Foote, L. E., Farley, H., and Wulf, M.
The spleen and Anaplasmosis. No. Amer. Vet. 32(12):827-828.
1951.
23. Foote, L. E., Levy, H. E., Torbert, B. J., and Oglesby, W. T.
Interference between Anaplasmosis and Eperythrozoonosis in
splenectomized cattle. Amer. Jour. Vet. Res. 18(68):556-559.
1957.
24. Franklin, T. E., and Redmond, H. E.
Observations on the morphology of *Anaplasma marginale* with
reference to projections or tails. Amer. Jour. Vet. Res.
19(70):252-253. 1958.
25. Gates, D. W., Roby, T. O., and Mott, L. O.
Specificity of the complement-fixation test for anaplasmosis.
Vet. Med. 51:5-6. 1956.
26. Gilbert, S. J.
A preliminary communication on the occurrence of Anaplasmosis
in sheep and goats in Palestine. Jour. Comp. Path. and Therap.
39(3):246-247. 1926.
27. Gledhill, A. W., and Dick, G. W. A.
The nature of mouse hepatitis virus infection in weanling mice.
Jour. Path. and Bact. 69:311-320. 1955.
28. Gledhill, A. W., and Niven, J. S. F.
The toxicity of some bacterial filtrates for mice pre-infected
with *Eperythrozoon coccoides*. Brit. Jour. Expt. Path. 38(3):
284-290. 1957.
29. Hoch-Ligeti, C., Irvine, K., and Sprinkle, E. P.
Investigation of serum protein patterns in patients undergoing
operation. Soc. Exptl. Biol. and Med. Proc. 84(3):707-710.
1953.
30. Hoffman, W. S.
The biochemistry of clinical medicine. 2nd ed. Chicago: Year
Book Publishers, 1959. pp. 33-51.
31. Jensen, R.
Eperythrozoonosis in cattle and sheep of Louisiana, preliminary
report. La. Agr. Expt. Sta. Bull. 366. 8 pp. 1943.
32. Jensen, R.
Suspected Anaplasmosis in sheep. No. Amer. Vet. 36(2):117-118.
1955.
33. Koprowski, H., Richmond, G., and Moore, D. H.
Electrophoretic study of antiviral sera. Jour. Exptl. Med.
85(5):515-530. 1947.

34. Kuttler, K. L., and Marble, D. W.
The effects of freezing and storage at -20°C . on serum protein patterns of sheep as determined by paper electrophoresis. *Amer. Jour. Vet. Res.* 20(76):434-436. 1959.
35. Leland, S. E.
Blood and plasma volume, total serum protein, and electrophoretic studies in helminthic disease. *N. Y. Acad. Sci. Ann.* 94(1):163-182. 1961. Original not seen. Abstract in *Biol. Abs.* 38:575-576. 1962.
36. Lotze, J. C.
Variables and constants in experimental bovine Anaplasmosis and their relationship to chemotherapy. *Amer. Jou. Vet. Res.* 8(28):267-274. 1947.
37. Lotze, J. C., and Yiengst, M. J.
Eperythrozoonosis in cattle in the United States. *No. Amer. Vet.* 22(6):345-346. 1941.
38. Lotze, J. C., and Yiengst, M. J.
Studies on the nature of Anaplasma. *Amer. Jour. Vet. Res.* 3(8):312-320. 1942.
39. Littlejohns, L. R.
Eperythrozoonosis in sheep. *Austral. Vet. Jour.* 36(6):260-265. 1960.
40. Luetscher, J. A. Jr.
Biological and medical applications of electrophoresis. *Physiol. Rev.* 27(4):621-642. 1947.
41. Marsh, H.
Newsom's sheep diseases. 2nd ed. Baltimore: Williams and Wilkins, 1958.
42. Neely, R. A., and Neill, D. W.
Accuracy and reproductibility of paper protein electrophoresis. *Nature* 176(4470):33-34. 1955.
43. Neitz, W. O.
Eperythrozoonosis in sheep. *Onderstepoort Jour. Vet. Sci. and Anim. Indust.* 9(1):9-33. 1937.
44. Neitz, W. O.
Eperythrozoonosis in cattle. *Onderstepoort Jour. Vet. Sci. and Anim. Indust.* 14(1):9-28. 1940.
45. Neitz, W. O., Alexander, R. A., and DuToit, P. J.
Eperythrozoon ovis (sp. nov.) infection in sheep. *Onderstepoort Jour. Vet. Sci. and Anim. Indust.* 3(2):263-269. 1934.

46. Ortega, L. G., and Mellors, R. C.
Cellular sites of formation of gamma globulin. *Jour. Exptl. Med.* 106(5):627-639. 1957.
47. Post, G., and Thomas, G. M.
A study of Anaplasmosis in elk. *Jour. Amer. Vet. Med. Assoc.* 139(3):357-358. 1961.
48. Quinlan, J., DeKock, G., and Marais, I. P.
The operation of splenectomy in horses, cattle, sheep, goats, pigs, dogs and some South African antelopes: a summary of the results of 98 splenectomies. *Onderstepoort Jour. Vet. Sci. and Anim. Indust.* 5(1):273-303. 1935.
49. Rees, C. W.
Splenectomy in cattle and sheep in relation to Anaplasmosis. *No. Amer. Vet.* 14(4):23-31. 1933.
50. Rees, C. W., and Underwood, P. C.
Comparative counts of infected and non-infected erythrocytes in bovine Anaplasmosis. *Helminthol. Soc. Washington Proc.* 6(2):48-50. 1939. Original not seen. Abstract in *Biol. Abs.* 14:719. 1940.
51. Ristic, M.
Structural characterization of *Anaplasma marginale* in acute and carrier infections. *Jour. Amer. Vet. Med. Assoc.* 136(9):417-425. 1960.
52. Ristic, M.
Studies in Anaplasmosis. III. An autoantibody and symptomatic macrocytic anemia. *Amer. Jour. Vet. Res.* 22(90):871-876. 1961.
53. Ristic, M., and Sippel, W. L.
Effect of Cortison on the mechanism of *Anaplasma* immunity in experimentally infected calves. II. Studies of pathological changes. *Amer. Jour. Vet. Res.* 19(70):44-50. 1958.
54. Ristic, M., White, F. H., Green, J. H., and Sanders, D. A.
Effect of cortisone on the mechanism of *Anaplasma* immunity in experimentally infected calves. I. Hematological and Immunoserological studies. *Amer. Jour. Vet. Res.* 19(70):37-43. 1958.
55. Ristic, M., White, F. H., and Sanders, D. A.
Detection of *Anaplasma marginale* by means of fluorescein-labeled antibody. *Amer. Jour. Vet. Res.* 18(69):924-928. 1957.
56. Ryff, J. F., Gilbert, C. S., Weibel, J. L., and Breen, H.
Anaplasmosis and concurrent copper intoxication in sheep. *Jour. Amer. Vet. Med. Assoc.* 133(6):312-315. 1958.

57. Simpson, C. F., and Sanders, D. A.
Diagnosis of the carrier stage of Anaplasmosis under experimental conditions. *Vet. Med.* 48(5):182-184. 1953.
58. Splitter, E. J.
Studies in Anaplasmosis II. 1938-1948. *Tech. Bull. Kan. Agric. Expt. Sta.* 66:1-24. 1948.
59. Splitter, E. J.
Eperythrozoon suis n. sp. and *Eperythrozoon parvum* n. sp., two new blood parasites of swine. *Science* 111(2889):513-514. 1950.
60. Splitter, E. J.
The complement-fixation test in diagnosis of *Eperythrozoonosis* in swine. *Jour. Amer. Vet. Med. Assoc.* 132(2):47-49. 1958.
61. Splitter, E. J., Anthony, H. D., and Twiehaus, M. J.
Anaplasma ovis in the United States. *Amer. Jour. Vet. Res.* 17(64):487-491. 1956.
62. Splitter, E. J., Twiehaus, M. J., and Castro, E. R.
Anaplasmosis in sheep in the United States. *Jour. Amer. Vet. Med. Assoc.* 127(942):244-245. 1955.
63. Stauber, L. A.
Application of electrophoretic techniques in the field of parasitic diseases. *Exptl. Parasitol.* 3(6):544-568. 1954.
64. Tiselius, A.
Electrophoresis of serum globulin. II. Electrophoretic analysis of normal and immune sera. *Biochem. Jour.* 31:1464-1477. 1937.
65. Twiehaus, M. J.
Research activities in Anaplasmosis. *Vet. Med.* 53(1):10-11. 1958.

OBSERVATIONS ON THE BLOOD ALTERATIONS ASSOCIATED WITH
EPERYTHROZONOSIS AND ANAPLASMOSIS IN SHEEP
FOLLOWING SPLENECTOMY

by

ARTHUR HARLAN BECKER

D. V. M., Kansas State University
of Agriculture and Applied Science, 1962

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Pathology
School of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1962

Twenty-three adult sheep, splenectomized at the beginning of the experiment, and two adult sheep which had been previously splenectomized were included in a study to determine the incidence of Eperythrozoon ovis and Anaplasma ovis infection. Hematological studies were conducted on the twenty-five animals to determine changes produced by relapse infections with either or both of these diseases in the carrier-infected sheep following splenectomy. One animal died as a result of the surgical procedure.

Packed cell volume and red blood cell determinations were recorded, and peripheral blood smears were prepared at regular intervals for 38 days following surgery. In addition, serum samples were collected and frozen for later use in the complement-fixation test and for serum protein determinations.

Eperythrozoon ovis was demonstrated in blood smears from the twenty-four sheep which survived. The organism was first observed 15 to 18 days following surgery.

Nine sheep reacted positively to the complement-fixation test for anaplasmosis, including two sheep which had been splenectomized prior to the beginning of the experiment. Anaplasma ovis could not be demonstrated in blood smears from the latter two animals, confirming that they were carrier-infected, but was noted to first appear 10 days after splenectomy in the other seven sheep.

Acute symptoms, on the basis of hematocrit and red blood cell determinations, occurred in one animal exhibiting infection with Eperythrozoon ovis and in one animal which relapsed with a dual infection with Eperythrozoon ovis and Anaplasma ovis. Symptoms in the other twenty-two sheep were relatively mild.

Increased severity of symptoms were noted when splenectomized sheep refractive to Eperythrozoon ovis infection were infected with Anaplasma ovis. Death occurred in two of three sheep so infected, whereas no mortality occurred in seven animals in which a simultaneous infection of Eperythrozoon ovis and Anaplasma ovis was demonstrated.

Electrophoretic studies were conducted on the sheep serums collected at weekly intervals during the experiment. A Spinco electrophoresis apparatus was employed, using a veronal buffer at pH of 8.6, 0.006 ml. of serum, and a current of 7½ milliamperes per cell for 10 hours. Relative protein values were converted to absolute values (gms./100 ml. serum) after the spectrophotometric determination of total serum protein.

Analysis of serum proteins revealed a pattern typical of acute infection in only one animal. Gamma-globulin showed an increase of more than 10% in nineteen of the twenty-four sheep studied, but a total globulin increase of greater than 10% occurred in only five animals. The occurrence of more than three peaks in the serum globulin fraction, and electrophoretic homogeneity in the latter part of the study, made the separation of distinct globulin fractions at times somewhat arbitrary.