Immunologic and Serologic Studies of Anaplasma Marginale.

Thomas Earl Rogers
Louisiana State University and Agricultural & Mechanical College

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IMMUNOLOGIC AND SEROLOGIC STUDIES OF ANAPLASMA MARGINALE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Bacteriology

by

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B.S., Northwestern State College of Louisiana, 1957
M.S., Northwestern State College of Louisiana, 1959
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ABSTRACT

An investigation was conducted to determine the activity and location of Anaplasma complement-fixing (CF) antibodies in serum protein fractions. The relationship between the concentration of the globulins and CF antibody titers was also studied. Anti-Anaplasma serums from calves infected with Anaplasma marginale were studied by electrophoretic separation techniques and CF. Fractionation of acute phase serums and subsequent serologic study of fractions showed that CF activity was associated with the alpha- and beta-globulins of lower mobility, and the gamma-globulin of highest mobility. Studies with convalescent serums showed a complete absence of CF activity in the alpha-globulin. The distribution of CF activity in the beta-globulin fractions was essentially similar to that found in acute phase serums although the titers were diminished. During the convalescent stages CF activity in the gamma-globulin was associated with fractions of high and intermediate mobilities. The apparent shift of CF activity may be due to inherent structural differences of the CF antibodies present in serum fractions which develop during acute and convalescent phases of the disease. These changes could account for differences in net charge as measured by electrophoretic methods and would be evidenced by changes in the location of CF activity. It was confirmed that a relationship existed between the concentrations of globulin components and CF antibody titers.
Studies were conducted to determine the nature of the Anaplasma CF antigen and to determine its relationship to the infected erythrocyte and the organism. Initial attempts were made to resolve the antigen from crude stromatal antigens by various chemical and physical methods of fractionation. Fractionation by partial and total lipid extraction suggested that the CF antigen was a lipoprotein. Fluorocarbon deproteinization of stromatal antigens was also attempted. Considerable color and undesirable stromatal material were extracted from these preparations but serologic activity was not increased.

Experiments were performed to determine the effects of sonic vibration on the serologic activity of stromatal CF antigens. Results showed that increased antigenic titers can be obtained by controlled sonic treatment.

A method was developed for the preparation of a highly desirable Anaplasma CF antigen. The method involved disintegration of infected erythrocytes by sonic vibration and separation of the antigen by differential centrifugation. Antigens prepared by this method were highly specific, colorless, did not exhibit anticomplementary activity and possessed much higher titers than standard Anaplasma CF antigens.

Qualitative biochemical tests and enzyme sensitivity studies indicated the antigen to be lipoprotein in nature. Electron microscopic observations of the antigen produced from sonicates of infected erythrocytes showed that the preparation was not composed of Anaplasma
bodies but of amorphous membranous material. The material reacted specifically when stained with fluorescent antibody.

Density gradient sedimentation was applied to sonicates of infected cells, and experiments showed that the CF antigen could be concentrated by this method.

Concentrated semi-purified Anaplasma bodies were prepared by differential centrifugation of sonicates of infected erythrocytes. The preparation of bodies was low in CF antigenic activity and in nucleic acid content, whereas a by-product consisting of a low-speed-sediment containing relatively few bodies, was high in CF activity and in nucleic acid content.

Data obtained using the CF test and the fluorescent antibody technique suggested that an antigenic matrix envelops the Anaplasma body and may be disrupted by physical means. This coat appeared to be responsible for the CF antigenic activity.
Anaplasmosis is an infectious noncontagious disease of cattle which has caused great economic losses to the American cattle industry as a result of decreased production and increased mortality. The infection is characterized by the presence of marginal inclusion bodies in erythrocytes and a progressive and often fatal anemia. Animals which survive the disease become carriers for life. Attempts to induce immunity against the disease by artificial means have not been successful.

Anaplasma marginale is the etiologic agent of anaplasmosis. The nature of the organism has been poorly understood and agreement on taxonomy has not been reached (Roby, 1960). The organism is classified as a rickettsia in Bergey's Manual of Determinative Bacteriology (Breed et al., 1957). Morphological evidence has been presented which also suggests classifying the organism as a virus (Foote et al., 1958) as well as a protozoa (DeRobertis and Epstein, 1951; Espana, 1957; Franklin and Redmond, 1958; Cane et al., 1963). Ristic (1960c) places Anaplasma somewhere between viruses and rickettsiae.

In addition to the disagreement on the classification of the organism the relative lack of knowledge concerning the nature of the etiologic agent as well as cellular and subcellular pathology in anaplasmosis has stimulated a great deal of basic research during the last seven years. Most of these studies have dealt with the
mechanism and nature of the anemia, pathology, development of the organism, and immunochemistry (Ristic et al., 1957; Hansard and Foote, 1958; Ristic and White, 1960; Ristic and Watrach, 1961; Ristic, 1960a; Dimopoullos and Bedell, 1960, 1961, 1962; Schrader and Dimopoullos, 1962; Cane et al., 1963).

The work reported in this dissertation concerns immunologic and serologic studies of the antibodies produced in anaplasmosis and the antigens of *A. marginale*. Experiments were designed to investigate changes in the serum proteins of calves infected with *A. marginale* and to determine the location and activity of complement-fixing (CF) antibodies in the serum protein fractions of animals in the acute and convalescent stages of anaplasmosis. Further studies were conducted to determine the nature of the CF antigen and to study its relationship to the infected erythrocyte and the infecting organism.
SELECTED LITERATURE

A. Historical Developments.

Anaplasmosis was first observed by Smith and Kilborne (1893a, b) in their studies of Texas fever of cattle. They considered the classical marginal body to be a stage in the life cycle of *Piroplasma bigeminum*, the causative agent of Texas fever. Dschunkowsky and Luhs (1904) studied the morphology of the organism and claimed it to be *Piroplasma annulatum*. Similar structures resembling the classical *Anaplasma* body (AB) were reported by Kolle (1898) and Lignieres (1914). Eventually, Theiler (1910a, b, c, d; 1911a, b) reported that mixed infections were present and that these bodies represented a new genus which he believed to be protozoan in nature. He named the organism associated with the bovine species *A. marginale*, indicating the absence of cytoplasm and the position of the body within the infected erythrocyte. Theiler's work and tentative classification of the organism was widely accepted and has received considerable support as a result of subsequent work by other investigators (du Toit, 1934; Sieber, 1911a, b; De Kock and Quinlan, 1926; Lotze and Yiengst, 1942; Lotze, 1946; Franklin and Redmond, 1958; Espana et al., 1959; Cane et al., 1963). However, classification of the organism still remains controversial (Roby, 1960; Ristic, 1960c).

Anaplasmosis exists in all of the tropical and subtropical countries as well as in many of the temperate areas of the world (Ristic, 1960c). The disease was first reported in the United States
by Meyer (1913) and has subsequently been reported in forty of the states (Ristic, 1960c).

Darlington (1926) was the first to give a clinical description of a natural case of bovine anaplasmosis in the United States. The symptoms of the disease are anemia, weakness, increased temperature, constipation, icterus, loss of appetite, reduced milk production, dehydration, labored respiration, and irrational behavior (Carricaburu, 1957a, b). The disease is generally more severe in adult animals than in calves (Christensen, 1956).

Anaplasmosis is reportedly transmitted by various species of ticks and horse flies, one species of stable fly, and three species of mosquitoes (Christensen, 1956). Stiles (1942) demonstrated experimental transmission of the disease by ticks. A recent review (Ristic, 1960c) indicates that 20 species of ticks may be involved. Direct transfer of blood from infected to susceptible animals by way of contaminated instruments, and improper surgical technique may bring about transmission of the agent.

For many years diagnosis of anaplasmosis was made solely on the basis of microscopic examination of stained blood films which demonstrated the presence of AB in the erythrocytes. Only in recent years has serologic diagnosis been developed to a somewhat reliable stage (Gates et al., 1949; Gates and Roby, 1956). The most significant advance was the development of the CF test for anaplasmosis (Gates et al., 1955).

Treatment of the disease has been studied by a number of workers but little success has been attained (Dykstra et al., 1938, 1948; Smith and Howell, 1944; Farley et al., 1949a, b; Foote et al., 1949;
Bedell and Oglesby, 1961). However, Aureomycin and Terramycin, when administered early in the course of the disease, have been shown to inhibit the growth of A. marginale (Miller et al., 1952, 1953). A successful immunizing agent or antiserum for anaplasmosis has not been developed.

B. Plasma Protein Changes.

A search of the literature has revealed only three reports of studies dealing with the plasma proteins of animals infected with A. marginale. Piettre (1943) did not find significant changes in the serum globulin and albumin fractions of animals in the acute stages of anaplasmosis. A decrease in the concentration of serum globulins was observed in samples obtained during the convalescent period. Rossi et al. (1956) reported that no qualitative changes occurred in the serum proteins of Anaplasma-infected animals. In a study conducted by Dimopoullos et al. (1960), it was shown that a decrease in the concentrations of all serum components occurred just prior to the appearance of marginal bodies within the erythrocytes. During the acute phase of infection the concentrations of alpha- and beta-globulins and total serum proteins increased while the amount of gamma-globulin was low. During convalescence, the concentration of gamma-globulin increased and the concentrations of alpha- and beta-globulins decreased to preinfection levels. It was suggested that the CF antibodies might be associated with the alpha- and beta-globulins early in the disease and appear later in the gamma-globulins. It was the latter work which stimulated the serum protein and antibody studies presented herein.
The scarcity of literature concerning the serum proteins of Anaplasma-infected animals and the questionable classification of the etiologic agent prompted the following review of literature on the response of the plasma proteins in infectious diseases.

Changes in plasma proteins of diseased and immunized animals are usually evidenced by a decrease in the albumin/globulin (A/G) ratio. Within the globulins the most striking change is an increase in the amount of gamma-globulin. In many cases, this increase in globulins is associated with the production of antibodies and a rise in antibody titer.

According to Luetscher (1947), the plasma globulins respond to infection in two distinct general patterns. In the febrile stage of acute infections, an increase in the amount of alpha-globulin usually occurs. This rise is accentuated by a reduction in the concentration of albumin. A change in the serum globulins, particularly in the gamma-globulin, follows in most infectious processes.

Petermann (1961) agrees with Luetscher and has classified the alterations in plasma proteins in bacterial infections into three types. Initially, there is a reaction to acute infection. The second change is a reaction to chronic infection, correlated with antibody production. While these are quantitative changes, the third type of change manifests itself in the appearance of C-reactive protein, a qualitative response to disease or injury.

In consideration of specific conditions and abnormal states, a number of studies have been selected to demonstrate the various changes that can occur during bacterial infections and immunization regimens. Studies of plasma protein alterations in syphilis showed
a decrease in albumin and some increase in all globulins soon after infection. If the disease remained untreated, these changes would be evident throughout the infection (Gutman, 1948). Electrophoretic studies of serum proteins in calves immunized with *Brucella* antigen showed a slight temporary increase in the concentration of gamma-globulins (San Clemente and Huddleson, 1943). Similar studies of the serum proteins in leprosy revealed a marked rise in alpha- and gamma-globulins (Seibert and Nelson, 1943). San Clemente (1942) studied the electrophoretic mobilities and composition of pullorum-agglutinating chicken serums and found a decrease in the A/G ratio. Alterations in electrophoretic mobilities were not noted. An increase in the concentration of gamma-globulin was reported by Lynch and Stafseth (1954), who studied pullorum-immune turkey serum. A number of studies have dealt with changes in the plasma proteins in tuberculosis. Seibert and Nelson (1942) and Seibert et al. (1947) found the usual decrease in the A/G ratio with the progressive development of the disease. Changes in the globulins were demonstrated by an increase in the alpha-globulins and the appearance of an additional component with a slightly greater electrophoretic mobility than albumin. It was suggested that these changes were associated with sensitization to the tuberculin protein. As the disease reached the terminal stage the amount of beta-globulin increased. A slight increase in gamma-globulin was correlated with antibody production. Ebel (1953) reported similar results in tuberculosis of dogs. Volk et al. (1953) studied approximately 100 cases of pulmonary tuberculosis and found elevations of the gamma-globulin to be of diagnostic significance when used in conjunction with other clinical tests.
Similar studies in rabbits, guinea pigs and rats showed the changes to vary between species (Hudgins et al., 1956; Hudgins and Patnode, 1957). An investigation of the plasma protein changes in rabbits infected with pneumococci (Jacox and Feldmahn, 1956) revealed significant alterations. An increase in alpha-globulin and fibrinogen accompanied a decrease in the concentration of albumin and beta-globulin. According to these authors, the degree of change in the plasma proteins appeared to be related to the severity of infection and to host response since the administration of penicillin decreased the degree of change in the plasma protein profile.

The literature is much less abundant in reports dealing with the plasma proteins in rickettsial infections. The response of the protein components, however, is similar to that which occurs in bacterial infections. For example, in a study of the serum proteins of a patient with typhus fever, Dole et al. (1947) found the relative concentration of albumin and the A/G ratio to decrease as a result of a relative increase in the concentration of gamma-globulin. The alpha- and beta-globulins did not change significantly. A greater increase in gamma-globulin was found in convalescent serum. Serum protein studies in dogs infected with Rickettsia canis showed an increase in the concentration of globulins while a decrease occurred in the concentration of the albumin fraction (Polson and Malherbe, 1952).

The changes in the plasma proteins associated with viral infections is somewhat in contrast to the dynamic state which prevails in other types of infectious diseases. In general, most viral infections result in little or no change in the profile of the plasma
proteins, according to a recent review (Dimopoullos, 1961). This lack of significant change was explained on the basis that the weights of antibodies associated with a plasma protein component are not great enough to cause an increase in any one particular component when the fractions are separated electrophoretically or by chemical means, even though the serum may possess high antibody activity. It has been suggested that in cases where decreases in the A/G ratio have been found the infections have caused a debilitating effect on the host and the capability of synthesizing albumin by the liver may be decreased (Dimopoullos, 1961).

A number of studies have been performed on serum proteins in virus hepatitis (Gray and Barron, 1943; Rabat et al., 1943; Moore et al., 1945; Martin, 1946; Ricketts and Sterling, 1949). All of these studies showed diminished concentrations of albumin, elevated gamma-globulin and in most cases elevated beta-globulin. It was suggested, however (Ricketts and Sterling, 1949), that the hepatic involvement in virus hepatitis may influence the serum protein patterns. Luetscher (1947) has pointed out that the pattern of the plasma proteins often reflects the total physiology and clinical state of the patient rather than a specific disease process.

Alterations in the plasma proteins have also been reported in protozoal diseases. In human malaria the albumin fraction was found to be diminished by 14.5 per cent, whereas the alpha\textsuperscript{1}-, beta-, and gamma-globulins increased 62, 20, and 19 per cent, respectively (Taylor et al., 1949). Significant changes in total protein
concentration were not found. In a study of the serum protein of chickens infected with malaria, decreases in the amount of albumin and total serum protein were observed (Rao and Cohly, 1953). The alpha- and gamma-globulins were elevated, while a significant change did not occur in the beta-globulin. It was concluded that liver and kidney damage as a result of the disease influenced the changes. Studies of the serum proteins in trypanosomiasis in guinea pigs (Ganzin et al., 1952) and in human leishmaniasis (Sen Gupta et al., 1953; Silver et al., 1961) showed an increase in serum globulins, primarily in the gamma-globulin fraction.

For more comprehensive information concerning the plasma proteins in disease, reference is made to reviews on this subject (Gutman, 1948; Jencks et al., 1956; Lewis et al., 1950; Luetscher, 1947; Marrack and Hoch, 1949; Stern and Reiner, 1946; Petermann, 1960, 1961; Dimopoullos, 1961, 1963).

C. Antibodies in Serum Protein Fractions.

The distribution of antibodies in serum protein fractions has received considerable attention. There are at least eight reviews (Kabat, 1943; Treffers, 1944; Campbell, 1948; Williams, 1950; Mayer, 1951; Smith and Jager, 1952; Dimopoullos, 1961, 1963) which give consideration to the residence of antibodies in the serum globulins in connection with immuno-chemical investigations of the proteins of serum.

A great deal of effort has been made to fractionate serum into its constituent proteins (Abramson et al., 1942). These efforts, many of which have been highly successful, may be condensed into
three major methods, namely, chemical precipitation, ultracentri­
fugation and electrophoresis. It is clear that precipitation and
electrophoresis have played dominant roles in the development of
antibody-isolation techniques.

Early observations of the elevated globulin concentration
accompanying an increase in antibody content in serum of various
species stimulated much interest and led to considerable speculation
on the nature of antibodies. It was thought by many workers that the
entire globulin fraction of serum might be composed of antibodies.
However, Boyd and Bernard (1937) studied the quantitative changes in
antibodies and globulin fractions in serums of rabbits injected with
several antigens and showed that the great increase in globulins
was not attributable to the specific antibody produced. It was
concluded that no more than 35 per cent of the total globulin
constituted the antibodies.

Subsequent studies have demonstrated that antibodies are
distributed throughout the globulin components of serums, although
the majority of different antibodies are concentrated in the gamma-
globulin fraction (Enders, 1944).

Tiselius and Kabat (1939) studied the electrophoretic patterns
of several immune serums produced in the horse, rabbit and monkey.
The antibody in horse anti-pneumococcal serum was found to migrate
between the beta- and gamma-globulin components. It was further
found that in anti-pneumococcal serums produced in rabbits and
monkeys, the percentage of antibody in the serum and in the gamma-
globulin fraction could be determined by integration of the electrophoresis diagrams of absorbed and unabsorbed serums. Moore (1940)
found pneumococcal antibodies in the \textit{gamma}-globulin fractions of
horse serums. Northrop (1949) purified pneumococcal antibody from
horse serum by precipitation of the euglobulins by dilution with water.
This fraction possessed an electrophoretic mobility similar to that of \textit{gamma}-globulin.

Van der Scheer \textit{et al}. (1940) made electrophoretic analyses of
hyperimmune serums against 15 antigens of bacterial origin. In some
of these the presence of antibody was evidenced by an increase in the
concentration of \textit{gamma}-globulin. In others the appearance of antibody
was accompanied by the development of a new component, designated as
"T," that possessed a mobility between that of the \textit{beta-} and \textit{gamma-}
globulins. Hess and Deutsch (1949) studied the serum protein distribu­
tion of antibodies in a cow that had been immunized with \textit{Brucella
abortus}. Agglutinins and bacteriocidins to this organism were
found in both the \textit{gamma$_1$-} and \textit{gamma$_2$}-globulin fractions. Blocking
antibodies to \textit{B. abortus} appeared in the \textit{gamma}-globulin soon after
immunization. Upon continued antigenic stimulation, these anti­
bodies became associated with the \textit{beta}-globulin fraction (Glencur

A number of studies have been made in an effort to determine
which protein fraction of syphilitic serum contained the reagins
(Abramson \textit{et al}. , 1942). Results were inconclusive until Cooper
(1944) investigated the activity of electrophoretic fractions of
serum containing syphilis antibody and correlated the results of
serologic reactions with the serum components present in the sample.
Both the \textit{beta-} and \textit{gamma}-globulin fractions carried the Kahn and
Wassermann reagins. Another report (Davis \textit{et al}. , 1945) showed
the Wassermann antibody to have a mobility intermediate between the beta- and gamma-globulins.

Enders (1944) found most of the antibodies to bacteria in human serums in a fraction composed entirely of gamma-globulin. Baldwin and Iland (1953) showed the CF antibodies in the serum of tuberculosis patients to be contained in the gamma-globulin fraction.

Antibody studies in viral diseases have been emphasized in a recent review (Dimopoullos, 1961). It was pointed out that a single purified antiviral serum globulin fraction may possess multiple serologic or immunologic activity and that similar activity may reside in more than one serum fraction. The review should be consulted for information on the development and current status of antibody studies in viral diseases. The following examples cite a number of investigations conducted in this area.

In studying anti-influenza hyperimmune horse serum, Wyckoff (1945) found the antibody to be associated with the gamma-globulin component.

Koprowski et al. (1947) utilized the Tiselius electrophoresis method in a study of antiviral serums. Neutralization tests were employed as a method of testing the activity of the serum fractions. Serums of animals immunized against Japanese B encephalomyelitis, Venezuelan equine encephalomyelitis, and Western equine encephalomyelitis viruses were studied. Serum fractions containing gamma-globulin were protective in all cases. The Japanese B encephalomyelitis antibody was associated entirely with the gamma-globulin, while the Venezuelan and Western equine encephalomyelitis antibodies resided with the beta- and gamma-globulins. It was suggested that
in the case of the latter two, the antibodies probably possessed an average electrophoretic mobility between that of the beta- and gamma-globulins. A similar study of hyperimmune rabbit serums against Eastern and Western equine encephalomyelitis, St. Louis and Japanese B encephalomyelitis and rabies demonstrated antibodies almost exclusively in the gamma-globulin (De Boer et al., 1952).

Hess and Deutsch (1949) studied the distribution of antibodies in a cow that had been immunized with Newcastle disease virus. Neutralizing antibodies appeared in both the gamma\textsubscript{1} and gamma\textsubscript{2} globulin fractions. The antibodies responsible for hemagglutination-inhibition were concentrated in the gamma\textsubscript{1}-globulin fraction.

The serum proteins in infectious mononucleosis have been fractionated and examined for the presence of heterophile antibodies (Sterling, 1947). Antibodies were found predominantly in the gamma-globulin fraction of the serums but were not always confined to this fraction.

The distribution of antibodies against foot-and-mouth disease virus has also received attention (Dimopoullos, 1961). In fractions of guinea pig serums separated by continuous-flow electrophoresis, virus-neutralizing antibody activity was demonstrated in the beta- and gamma-globulins (Dimopoullos and Fellowes, 1958). Complement-fixing antibodies were found only in the gamma-globulin fraction of highest electrophoretic mobility. It was also shown that the complement-fixing and virus-neutralizing antibodies were distinctly different antibodies. Immunoelectrophoretic studies of guinea pig serums from animals recovering from the same disease showed that precipitating antibodies migrated from a position corresponding to
the beta-globulin seven days after infection, to a position corresponding to the gamma-globulin, at 14 days or later (Brown, 1960).

A search of the literature revealed little on the distribution of antibodies in rickettsial and protozoan infections. Pierce (1955) conducted electrophoretic and serologic studies on agglutinins to *Trichomonas foetus* in calf serum. Normal and induced agglutinins were demonstrated in the gamma-globulin fraction of the serums from the immunized animals.

D. Development of Anaplasma CF Antigen.

Many attempts have been made to prepare antigens for the CF test for anaplasmosis from various tissues of *Anaplasma*-infected animals (Gates *et al.*, 1954). Antigenicity of water-laked infected erythrocytes was first demonstrated by Mohler (1932), using the CF test. The first laboratory application of the CF test to the diagnosis of bovine anaplasmosis was made in 1934 when a tick antigen was developed. It was produced from the viscera of nymphs and adults of *Rhipicephalus sanguineus*, which were known to harbor the etiologic agent (Rees and Mohler, 1934). The limited quantity of this antigen did not permit experimental testing.

In 1944 a crude blood antigen was developed by the United States Bureau of Animal Industry (Gates *et al.*, 1954). The antigen could be produced in sufficient quantities, but it contained a large amount of hemoglobin, which interfered with interpretation of the test. It was also nonspecific and anticomplementary. Following this, a carbon dioxide-precipitated antigen was prepared by the Bureau (Gates *et al.*, 1954) using the technique of Heidelberger and Mayer (1944). This
procedure eliminated most of the color and resulted in a more uniform antigen. It still had the disadvantage of being anticomplementary. Price et al. (1952) produced a more suitable antigen from water-lysed erythrocytes. The antigen was less anticomplementary than previous antigen preparations and possessed a higher titer. Modification of this method of preparation by using the Sharples centrifuge allowed Miller (Gates et al., 1954) to produce the antigen more efficiently.

Gates et al. (1954) compared antigen production methods and CF testing techniques in a study where all serums and cells were derived from a common source. Antigens employed were a carbon dioxide-precipitated antigen and two antigens prepared from water-lysed erythrocytes concentrated by high speed centrifugation. In each preparation the product consisted of stromata of erythrocytes from *Anaplasma*-infected cows. The antigenicity of all three preparations was similar. All antigens possessed some degree of anticomplementary activity and color which made the CF test difficult to interpret. Although these antigens are employed at present for the serologic diagnosis of anaplasmosis, a more desirable CF antigen has not been produced nor have studies been performed which have revealed the nature of the CF antigen.

E. Purification of Antigens.

In anaplasmosis the CF test is the only serologic test which has been routinely used for diagnosis. Since the classification of *Anaplasma* is unsettled, methods which have been used in the preparation and purification of CF antigens of various organisms will be considered. Certain methods of purification of antigens other than
CF antigens will also be mentioned since the techniques employed were useful in the work presented herein.

In general, the techniques which have been most useful in the preparation and purification of antigens have incorporated the principles of sonic vibration, differential centrifugation, density gradient sedimentation, chemical fractionation, precipitation and/or extraction. A number of antigens have been prepared simply by centrifugal clarification of the fluids of tissue cultures in which viruses have been cultivated. Provided enough difference exists in the relative densities of the antigens and cellular debris, differential and density gradient centrifugation are very useful. This is especially true in cases where sonic vibration or pressure is used to liberate the agents from within host cells or in cases where microorganisms are fractionated to obtain cellular components which possess high antigenic activity. Removal of excess proteinaceous material in the purification of certain viral agents has been accomplished by use of the fluorocarbons (Manson et al., 1957). Soluble antigens generally may be purified by removing water-insoluble materials with organic solvents. For instance, ether treatment of yolk sacs containing rickettsiae of typhus fever (Topping and Shear, 1944; Shepherd and Wyckoff, 1946) resulted in the separation of a soluble CF antigen. Similar antigens were prepared from yolk sacs infected with Rocky Mountain spotted fever and rickettsialpox. Q fever rickettsiae in infected yolk sacs and chick embryo tissues were isolated and purified by differential centrifugation of homogenates followed by sedimentation in continuous linear gradients of sucrose and glycerin (Ribi and Hoyer, 1960).
CF antigens were prepared from central nervous tissue of mice infected with poliovirus (Cassals et al., 1951). Homogenized tissues were extracted with acetone and a mixture of acetone-ether. The dry residues were suspended in saline and centrifuged and the supernatant fluid constituted the antigen. Le Bouvier (1953) prepared a similar antigen without acetone-ether extraction. Purification of polioviruses from tissue culture fluids was accomplished by precipitation, butanol extraction, ultracentrifugation and treatment with nucleases followed by electrophoresis or sedimentation in a density gradient column (Schwerdt and Schaffer, 1956). Polioviruses have also been purified using fluorocarbon deproteinization techniques (Manson et al., 1957).

A number of other viral antigens have been prepared using similar methods. Influenza virus CF antigens have been prepared by differential centrifugation (Kirber and Henle, 1950). Soluble influenza CF antigen was prepared by extraction of chick-embryo lungs with methanol and chloroform (Ada et al., 1952). Foot-and-mouth disease virus was purified from tissue culture fluids by alcohol precipitation, extraction with organic solvents and differential centrifugation (Bachrach and Breese, 1958). Encephalomyocarditis virus was separated from infected mouse brain by protamine sulfate clarification followed by trypsin digestion and ultracentrifugation (Weil et al., 1952). CF antigens for mumps have been prepared by clarification of monkey parotid gland emulsions by centrifugation (Habel, 1945). Coxsackie viruses were purified from mouse muscle tissue and amniotic fluid by ether extraction, high speed centrifugation, and trypsin digestion (Briefs et al., 1952). CF antigens for trachoma virus were prepared
from ground, phenolized yolk sacs clarified by centrifugation (Woolridge and Grayston, 1962). A similar procedure, followed by high speed centrifugation, was used to produce a CF antigen consisting of purified elementary bodies. Lipoidal CF antigens have been purified from ether extracts of the psittacosis-lymphogranuloma group of viruses by fractionating with organic solvents (Hilleman and Nigg, 1946, 1948). Elementary body antigens of this group of viruses were purified by ether fractionation and differential centrifugation (Smadel et al., 1943).

Various methods have been described for preparing CF antigens of malaria (Russell et al., 1946). Eaton and Coggeshall (1939) prepared an antigen from dried erythrocytes infected with *Plasmodium knowlesi*. Dried cells were rehydrated with physiologic saline, the suspension frozen and thawed four times, and centrifuged. The resulting supernatant fluid constituted the antigen. A similar antigen was prepared from infected red blood cells, but the stromata were ground to a powder prior to freezing and thawing (Dulaney and Stratman-Thomas, 1940). This antigen was improved by extracting with phosphate or barbiturate buffers rather than with saline. The parasites may be liberated from infected erythrocytes by saponin treatment or by lysis with guinea pig complement and anti-erythrocyte hemolytic antibody (Moulder, 1962). Spira and Zuckerman (1962) made an erythrocyte-free preparation of the erythrocytic stages of *P. vinckei* by lysing infected erythrocytes with a saponin-saline solution and differential centrifugation.

In addition to these microbial antigens it is appropriate to mention studies on fractionation of erythrocytes and characterization
of the blood group factors. Calvin et al. (1946) separated from red blood cell stromata a lipoprotein called elinin which yielded an ether-soluble fraction possessing Rh activity. A later communication described the separation of elinin and showed the fraction to contain Rh, A and B factors (Moskowitz et al., 1950). The techniques employed which involved extraction with ether and differential centrifugation have been of importance in the present study of infected erythrocytes.
MATERIALS AND METHODS

A. General.

1. Experimental Calves.

Experimental calves were obtained from local livestock auctions, maintained in closed, screened housing and isolated or separated into small groups. Calves were of mixed breed and sexes and ranged from three days to three months of age. Nutrition and care of calves were supervised by a graduate veterinarian and two husbandrymen. All animals were splenectomized at least two weeks prior to inoculation. Rees (1933) showed that calves less than one year of age were more susceptible to anaplasmosis after splenectomy.

2. Blood Collection and Inoculation.

Blood was obtained three times weekly from all calves to establish individual, normal hematologic values and to detect and follow Anaplasma infections. Five milliliters of blood were collected by venipuncture in tubes containing ammonium and potassium oxalates (Hepler, 1957) or 25 mg of dipotassium ethylenediamine tetraacetate as anticoagulants. Blood samples were collected from experimentally-infected animals throughout the acute phase of the disease until the animals either died, recovered, or were exsanguinated. Blood used for the collection of erythrocytes and subsequent preparation of experimental antigens was collected in flasks containing heparin sodium solution (1,000 U. S. P. units/ml) at the rate of 0.3 ml/50 ml blood. In cases where serum was collected it was separated by centrifugation after clot retraction.
Ten milliliters of blood from calves in the acute stages of infection or from carrier animals were inoculated into splenectomized calves by the subcutaneous route to produce the disease.

3. Hematology and Detection of Infection.

The incubation period required for infection was usually seven to 21 days. Infection was diagnosed by the presence of typical marginal bodies in at least one per cent of the erythrocytes in the peripheral blood of the animals. This was determined by microscopic examination of Giemsa-stained, thin films of blood. Red blood cell counts and packed cell volumes were routinely determined in order to follow the anemia during the course of the disease.

B. CF Antibody.

Blood samples were obtained from 30 infected calves at various times throughout the acute and convalescent stages of the disease and from 18 normal animals. The serums were stored at -20 C until they were used for study.

1. Selection of Serums.

Serum samples from calves in the acute phase of the disease were selected on the basis of high AB counts and severe anemia. Convalescent serums were selected from animals that were showing an increase in the number of erythrocytes in their peripheral blood and possessed no AB or a low AB count. This usually occurred at approximately 40 to 50 days after initial infection. Serums were tested for CF antibodies according to the methods outlined for the serologic diagnosis of anaplasmosis (United States Department of Agriculture, 1958).
2. **Serum Proteins.**

All serum samples obtained for antibody studies were analyzed for absolute concentrations of albumin, and **alpha-, beta- and gamma-** globulins. Total protein content was determined by the biuret method as modified by Weichselbaum (1946) and serum protein composition by paper strip electrophoresis.

a. **Paper strip electrophoresis.**

Serums were separated for analysis by paper electrophoresis using a Durrum type, vertical electrophoresis cell (Model R, Spinco). Undiluted specimens were separated in barbital buffer, pH 8.6, 0.075 ionic strength. One-hundredth ml samples were applied to the paper strips after proper stabilization of buffer and current. A current of five milliamperes was applied for 16.5 hours at 25 C. The paper strips were dried at 125 C for 30 minutes after which they were dyed with bromphenol blue (Block *et al.*, 1955). After the strips were redried they were scanned with a densitometer-integrator (Analytrol, Spinco) to determine relative percentages of serum protein components. The absolute protein concentration of each serum fraction was calculated from the values obtained for total serum protein and relative percentages.

3. **Fractionation of Serums.**

a. **Continuous-flow paper electrophoresis.**

Serum fractionations were made using a continuous-flow paper electrophoresis apparatus (Model CP, Spinco). In each case involving the fractionation of normal, acute and convalescent serums, all samples were diluted 1:2 with veronal buffer solution, pH 8.6, ionic strength 0.025. The same buffer was also used as the
electrolyte for all such analyses. After the electrophoresis cell was equipped with wicks, curtains and buffer, a current of 93 milli-amperes was applied, and the system was allowed to equilibrate for at least nine hours before the sample was applied. One hundred and ten milliliters of the serum-buffer mixture were allowed to flow on the paper curtain (S & S 470) at a rate of approximately one ml per hour for a total of 96 hours. The electrophoretic cell was maintained at approximately 1 C during all fractionations. An average total of 22 fractions which contained protein was collected in tubes from drip-points along the paper curtain using an automatic fraction collector (Fraction Collector, Spinco). All fluids originating from the same drip-point were pooled at the end of fractionation and kept at 4 C until they were dialyzed and concentrated. The paper curtains were dried at 125 C for 30 minutes and stained with bromphenol blue to locate the protein bands and their respective drip-points.

b. Preparation of fractions.

The fractions that originated from the same drip-point throughout the fractionation procedure were pooled and dialyzed against several changes of distilled water to eliminate veronal salts. This was determined with a Leeds and Northrop conductivity bridge. Dialysis was continued against 15 per cent aqueous poly-vinylpyrrolidone for 48 hours at 8 C to remove most of the water. The contents of each dialysis bag were further concentrated by freeze-drying. The dried serum protein fractions were reconstituted with sufficient 0.85 per cent sodium chloride solution at the rate of approximately two ml for each fraction to give the same total volume of serum that had flowed on the curtain from the sample reservoir.
More adequate estimates of antibody and protein content in each fraction could be made in this manner.

   a. Protein analysis.

   Total protein determinations were conducted on all serum fractions using the biuret method in order to calculate per cent protein recovered. Each fraction was identified and analyzed for homogeneity by paper strip electrophoresis as previously described.

   b. Antibody assay.

   Aliquots from each serum fraction were assayed for Anaplasma CF antibody activity employing the method previously described with one exception. In order to eliminate the anti-complementary activity which is usually encountered in concentrated serum fractions (Davis et al., 1944; Beck, 1960; Marcus, 1960) the samples were initially diluted 1:5 with normal bovine serum rather than with the veronal-sodium chloride buffer solution (CF buffer) which is ordinarily employed in the CF test. The diluted samples were then inactivated in a water-bath at 58°C for 35 minutes, and further dilutions were made with the CF buffer to determine CF antibody titers. Appropriate controls of positive and negative serums, serum fractions, antigen, complement and sheep red blood cells were included in all tests.

C. CF Antigen.

Blood used for the preparation of CF antigens was obtained from Anaplasma-infected calves with 50 per cent or more of the erythrocytes containing AB with the following exception. When the relationship of
antigenicity to number of erythrocytes containing AB was studied, blood with lower percentages of AB was used. Blood cells were sedimented by centrifugation, the plasma and white cell layers were removed by aspiration, and the remaining erythrocytes were washed with 0.85 per cent sodium chloride solution at least three times by centrifugation at 700 x g for 30-minute intervals at 4 C. Packed cells were resuspended in an equal volume of saline and stored at 4 C if not used immediately. If storage was for more than a few hours, cells were rewashed before use.

1. Preparation of Stromatal Antigens.

Washed, packed red blood cells from 12 Anaplasma-infected animals and six normal animals were lysed separately in 20 volumes of carbon dioxide-saturated distilled water per volume of packed cells at 4 C. The carbon dioxide-saturated water possessed a pH of 3.8. The stromata were allowed to precipitate in flasks or tall cylinders overnight at 4 C. The supernatant fluid was aspirated, and the stromata were washed in distilled water, pH 5.4, at 700 x g for 10-minute intervals at 4C. The material was washed until no red color, due to hemoglobin, was visible in the supernatant fluid. After the last washing the supernatant fluid was poured off, the volume of packed stromata measured, and separate pools were made and designated normal stromata and Anaplasma stromata. Stromata were diluted 1:1 with distilled water and freeze-dried. For the determination of antigenic titer, aliquots of the stromatal pools were diluted 1:10 with CF buffer. CF antigenic titers were determined according to the procedure mentioned earlier.
a. Fractionation of stromatal components.

Dried stromata were fractionated by the procedure outlined in Figure 1. The techniques were similar to those which have been used (Jorpes, 1932; Calvin et al., 1946; Moskowitz et al., 1950, 1952) in the separation of the components of human red blood cell membranes. In preparing fractions for determination of antigenic activity, solvents were removed using a flash evaporator. The residues and other fractions were mixed with minimal volumes of CF buffer, the exact volume depending upon the amount of fraction recovered. Fractions were tested for antigenic activity using standard anti-Anaplasma and normal serums.

b. Extraction with lipid solvents.

One-step extraction was also made with lipid solvents. Five milliliters of stromata, diluted 1:10 with CF buffer, were added to 200 ml of a 3:1 alcohol:ether solution and mixed overnight at 4 C. The mixtures were centrifuged for 15 minutes at 400 x g and supernatant fluids were collected. Ether and alcohol were removed using a flash evaporator, leaving aqueous suspensions of the extracted material. Residual solvents were evaporated from the sediments in the same manner. One milliliter of the sediments was suspended in five ml of CF buffer and titrated for antigenic activity.

c. Extraction with fluorocarbon.

One hundred and eighty milliliters of Genetron 113, 18 ml of stromata and 72 ml phosphate buffer solution pH 7.2, were mixed and homogenized for five minutes at 16,000 rpm in an Omnimixer at 4 C (Gessler et al., 1956a, b; Porter, 1956). After homogenizing, the emulsion was broken by centrifugation for 10 minutes at 1000 x g.
Figure 1. Diagrammatic scheme for fractionation of stromata.
The aqueous layer was removed and tested for CF antigenic activity without further treatment. Multiple extractions by the same techniques were also made on additional stromata.

A second technique (Halonen et al., 1958) was employed whereby 20 ml of stromata were exposed to sonic vibration for 10 minutes. Afterwards, the material was mixed with 10 ml of the Genetron and homogenized as before. The homogenate was centrifuged for 10 minutes at 700 x g. One milliliter of the aqueous layer was removed, diluted 1:6 with CF buffer and titrated. The procedure was repeated on another portion of stromata, but the aqueous layer was centrifuged for 90 minutes at 105,000 x g. A brown supernatant fluid was removed, and the pellet was suspended in 10 ml of CF buffer. Both the supernatant fluid and the suspension of sediment were tested for CF antigenicity.

d. Electrophoresis.

In order to test the feasibility of electrophoretic separation of stromata, samples were subjected to paper strip electrophoresis. Aliquots of normal and infected stromata were diluted 1:5 in distilled water and in the buffer used as the electrolyte. Michaelis buffer solutions were employed and separations were attempted at pH 2.2, 2.3, 4.2, 4.7, 5.25, 6.42, 7.3, 7.77, 8.25, 8.69, and 9.0, at a current of five milliamperes. Otherwise the technique used was the same as that previously described.


Stromatal CF antigens, prepared as previously described, were titrated according to protocol. Samples of the antigens were subjected to sonic vibration at 17 to 20 C using a 50 watt, 9-kc
oscillator (Model S102A, Raytheon Magnetostriction Oscillator). The antigens were treated in 25 ml quantities for 1, 2, 5, 10 and 15 minutes. Following treatment, all samples were titrated for CF antigenic activity.

2. Production of CF Antigen by Sonic Treatment.

Red blood cells from acutely-infected animals were separated and washed according to the procedure previously described. Packed cells were suspended in an equal volume of isotonic phosphate buffer solution, pH 7. The suspension was subjected to sonic vibration for five minutes under the conditions specified earlier. The sonicate was centrifuged at 700 x g for 30 minutes, the supernatant fluid recovered, and centrifuged for one hour at 105,000 x g and 1 C. The resulting sediment was washed in phosphate buffer, pH 7, at 105,000 x g for one-hour intervals until the supernatant fluid did not contain red color due to hemoglobin. After the last washing, the sediment was resuspended in CF buffer and titrated along with the first high speed supernatant fluid, according to protocol for CF antigen titration.

a. Filtration.

Antigen was prepared according to the procedure described above and subjected to serial filtrations in order to determine the approximate size of the antigenic material. The final sediment was suspended in 50 volumes of phosphate buffer and initially passed through a microfiber glass prefiltet (Millipore Filter Corporation). After each filtration a five-ml sample of the filtrate was set aside while the remainder was passed through the next filter in the sequence. Subsequent filtrations were performed using 5000, 3000, 1200,
650 and 450 μm pore sizes. Residues were washed once with phosphate buffer after each filtration was completed. Filtrates were centrifuged for 90 minutes at 105,000 x g, and the sediments were resuspended in five ml of CF buffer and titrated for CF antigenicity.

b. Absorption.

Experiments were performed to determine whether an antigen-antibody complex would form and be evident as an alteration in the serum protein pattern when CF antigen and antiserum were mixed. Undiluted antigen was mixed with normal and anti-Anaplasma serum in a ratio of 0.2 ml antigen to 0.5 ml serum. The mixtures were incubated for one hour at 37 C and for six hours at 4 C. Appropriate controls were treated likewise. After incubation the mixtures were subjected to paper strip electrophoresis according to the technique described earlier. The dyed strips were examined for evidence of an antigen-antibody reaction.

c. Antigenicity and AB count.

In order to determine the relationship of CF antigenicity to the number of erythrocytes containing AB, blood was obtained from normal and carrier calves and from calves in the acute stages of the disease with 6.5 to 90 per cent of the erythrocytes containing AB. Two-hundred ml of packed cells from each animal were used in preparing separate antigens by the method described above. Aliquots of each preparation were titrated, and CF antigenicity was compared.

d. Microscopic examination.

Smears of the undiluted antigen were prepared and stained with Giemsa stain. Uranium-shadowed preparations of the antigen were also examined by electron microscopy.
e. Biochemical characteristics.

Since this antigen possessed higher CF activity than any other preparation, it was of interest to make certain qualitative chemical determinations. Tests for protein were performed using the xanthoproteic and Millon's tests, and the anthrone and Molisch tests were used for the detection of carbohydrate. Tests for cystine were made by heating a mixture of lead acetate and a sample of antigen dissolved in 1N sodium hydroxide (Harrow et al., 1955). The presence of cholesterol was determined by the Salkowski and Liebermann-Burchard tests. Lipid analyses were made using the thin-layer chromatography technique (Mangold, 1961) to detect classes of lipids. Quantitative protein determinations were also made using the biuret test.


The antigen was exposed to various enzymes to determine their effects upon the CF antigenic substance. The enzymes, pH, enzyme concentration and the activator used are given in Table I. Antigen was diluted 1:5 with CF buffer, distributed in five ml aliquots, and enzymes were added to give the final concentrations indicated in Table I. The mixtures of antigen and enzymes were incubated for one hour at 37 C and then five hours at 4 C. The treated antigen samples were sedimented by centrifuging for 20 minutes at 39,000 x g and washed three times with CF buffer by the same process. The washed sediments were reconstituted to five ml with CF buffer and titrated.

g. Effects of temperature.

Antigen was incubated in a water-bath for two hours at 60 C. Additional samples remained at 25 C for 12 hours and others
Table I. Enzymes employed to treat *Anaplasma* CF antigen and conditions of treatment.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Concentration (%)</th>
<th>pH</th>
<th>Activator (0.1M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>0.35</td>
<td>8.2</td>
<td>Ca^{++}</td>
</tr>
<tr>
<td>Taka-diastase</td>
<td>0.75</td>
<td>7.0</td>
<td>none</td>
</tr>
<tr>
<td>Phospholipase A</td>
<td>0.10</td>
<td>7.0</td>
<td>Ca^{++}</td>
</tr>
<tr>
<td>Lipase</td>
<td>0.75</td>
<td>7.6</td>
<td>Ca^{++}, Mg^{++}</td>
</tr>
<tr>
<td>DNAase</td>
<td>0.50</td>
<td>7.0</td>
<td>Mg^{++}</td>
</tr>
<tr>
<td>RNAase</td>
<td>0.50</td>
<td>7.0</td>
<td>none</td>
</tr>
</tbody>
</table>

* Taka-diastase obtained from Parke, Davis and Co.; all other enzymes obtained from Nutritional Biochemicals Corp.
were frozen and thawed several times. Titrations were performed on all samples to determine the effects of these temperature treatments on the CF antigenic activity.

3. Preparation by Differential Centrifugation.

A method (Dintzis et al., 1958) which has been successful in the separation of reticulocyte microsomes was applied to erythrocytes infected with AB. An outline of the entire procedure is given in Figure 2. The final pellet was suspended in 10 ml of CF buffer and titrated.

4. Purification of Anaplasma Bodies.

In order to test the CF antigenicity of the AB it was necessary to obtain AB in a concentrated and reasonably purified state. A method was developed for the preparation of highly concentrated AB containing only small amounts of erythrocyte material. An outline of the procedure appears in Figure 3. A similar method was recently described by Ristic (1962) for the preparation of a capillary agglutinating antigen which consisted of purified AB. This method was also tested and compared with the above procedure. All fractions obtained in the processes were tested for CF antigenicity and examined microscopically for the presence of AB. Further qualitative determinations on a number of biochemical constituents were made according to the methods outlined in section 2 b. In order to test infectivity and in vivo antigenicity, susceptible calves were inoculated with suspensions of the material containing AB and the final sediment. These fractions also were analyzed for concentrations of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) (Volkin and Cohn, 1954) and protein. Ribonucleic acid was extracted by the
Red Blood Cells
(1:1 in 0.85% NaCl)

Sonic vibration,
5 minutes

Sonicate mixed with 3 volumes
cold buffer solution
(0.14M KCl, 0.01M MgCl₂,
0.01M tris chloride, pH 7.2)

700 x g,
30 minutes

Sediment
(cell membranes)

Supernatant
fluid

105,000 x g, 3 hours

Sediment

Supernatant
fluid

Washed 2X in
100 volumes buffer solution

105,000 x g, 3 hours

Sediment

Supernatant
fluid

Figure 2. Diagrammatic scheme for preparation of *Anaplasma* CF antigen by method of purification of reticulocyte ribosomes (Dintzis et al., 1958).
Figure 3. Diagram of procedure for concentrating AB.

Washed Red Blood Cells
(suspended 1:1 in CF buffer)

Sonic vibration
12 minutes

700 x g, 30 minutes

Sediment
(intact cells)

Supernatant
fluid

20,000 x g, 20 minutes

Sediment

Supernatant
fluid

Resuspended 1:5 in CF buffer

Sonic vibration, 15 seconds

Washed 2X in 100 volumes CF buffer,
20,000 x g, 20 minutes

Sediment

Supernatant
fluid

Resuspended in 1:5 CF buffer

2,000 x g, 1 minute

Sediment

Supernatant
fluid
method of Wecker (1958) from the sediment found to contain large quantities of nucleic acids. One animal was inoculated with the entire extract to determine its infectivity and antibody response.

5. **Density Gradient Sedimentation.**

Blood from 12 calves was obtained when microscopic examination of Giemsa-stained blood smears demonstrated at least 72 and as high as 90 per cent of the erythrocytes to contain AB. Blood was collected by exsanguination in heparin solution as described. Erythrocytes were separated from plasma by centrifugation, washed three times with 0.85 per cent sodium chloride solution and pooled. White blood cells were removed by aspiration after each centrifugation. Pooled erythrocytes were washed once with CF buffer and resuspended in an equal quantity of the same buffer. The suspensions were subjected to sonic vibration for intervals of 10 minutes until the entire pool had been treated.

Gradients were prepared by layering 4, 7, 7, and 7 ml of veronal-buffered saline solution containing 600, 500, 400 and 300 mg sucrose/ml, respectively in 1 x 3 inch cellulose nitrate tubes. Two-ml quantities of the sonicate were layered over the gradient columns and the tubes were centrifuged at 50,000 x g for one hour in a swinging bucket rotor (SW-25.1, Spinco). Layers of the gradient were removed, from top to bottom, using a pipette with a curved tip drawn out to a small opening at the end. The procedure was repeated for greater purification of one layer which contained concentrated AB. Polyvinylpyrrolidone as well as sucrose were used in preparing linear gradient columns; however, this was found to offer no advantage over the technique described and was discontinued after initial testing.
a. **In vitro** tests of antigenicity.

All layers of fractionated material were titrated for CF activity according to protocol. In addition to the usual controls, sucrose solutions corresponding to those used in preparing the gradients were tested using standard antigen and serums. Comparisons were made of CF antigenic activity in the presence and absence of sucrose. Sucrose was removed by repeated washing in veronal-buffered saline at 50,000 x g for 30 minutes. Fractions were also tested for reaction with fluorescein-conjugated anti-*Anaplasma* globulin.

b. **In vivo** tests of antigenicity.

The effect of gradient materials on the infectivity of *A. marginale* was tested. After lysis of erythrocytes, equal volumes of the sonicate and 30 per cent sucrose solution were mixed and allowed to stand at 4°C overnight. A sample of the sonicate was likewise treated with 10 per cent polyvinylpyrrolidone. Susceptible calves were inoculated, each with 10 ml of the mixtures. A layer containing concentrated AB, produced by the sedimentation technique, was also inoculated. Control calves were inoculated with the untreated blood. Routine blood samples were examined for the presence of AB, and samples of serum were checked for *Anaplasma* CF antibodies.

6. **Plasma Sedimentation.**

An attempt was made to obtain CF antigenic material from the plasma of animals acutely infected with *A. marginale*. Three liters of plasma were separated from the blood of calves when the number of erythrocytes containing AB was greater than 50 per cent. The plasma was pooled and centrifuged in a continuous-flow centrifuge (RC-2, Servall) at 39,000 x g. The rate of flow was approximately 60 ml per
hour. The plasma was aspirated, the sediment suspended 1:5 in CF buffer and tested for CF antigenicity. Smears of the sediment were prepared and were examined after staining with Giemsa-stain.

**Immunologic and Structural Relationships of Anaplasma Bodies**

A. **Fluorescent Antibody Staining.**

1. **Preparation of Labelled Globulin.**

Serums with high CF antibody titer were obtained from calves with acute anaplasmosis. Fluorescent antibody was prepared according to methods outlined by Cherry et al. (1960) except for minor variations. Globulins were precipitated by half-saturation with ammonium sulfate and separated by centrifugation. The globulins were dialyzed at 4°C against 0.85 per cent sodium chloride solution until sulfate was no longer detectable in the dialysis fluid when tested with barium chloride.

The purified globulin was adjusted to one per cent protein concentration with 0.85 per cent sodium chloride solution using a serum protein meter (Serum Protein Meter, Bausch & Lomb). The globulin was chilled and mixed with 10 per cent by volume of carbonate-bicarbonate buffer (0.5M, pH 9.0). The mixture was placed in an ice-bath and 0.05 mg of fluorescein isothiocyanate was added per mg of protein in solution and stirred overnight at 4°C. The protein solution was dialyzed in 50 ml quantities, for 24 hours against 0.85 per cent sodium chloride containing about 20 grams of Dowex 2-X4, 20-50 mesh, anion exchange resin (Dow Chemical Co.) per bag. The dialysis solution was replaced with 0.01M phosphate-buffered saline and dialysis
was continued until it was determined, by staining of a standard sample of antigen, that excess fluorescein was removed. The fluorescein conjugated globulin (FCG) was mixed with 100 mg of buffered, normal bovine liver powder per ml of solution and allowed to stand at 4 C for one hour. FCG was recovered by centrifugation at 20,000 x g for 30 minutes. The solution was dispensed in small vials and kept at -17 C until used.

2. Staining.

Regular thin smears of specimens to be examined were air dried, fixed for five minutes with absolute methanol, rinsed in phosphate-buffered saline, pH 7.2 and blotted dry. Slides were flooded with FCG and incubated for one and one-half hours. After incubation, they were rinsed with buffered saline for 10 minutes, with tap water for an additional five minutes and blotted dry. A drop of buffered glycerol was added to the area to be examined and a cover glass placed onto the area covered by the drop.

3. Observation and Photography.

Stained specimens were examined under dark-field with ultraviolet and tungsten illumination and compared with standard control specimens. Controls consisted of known Anaplasma blood, stained with normal and anti-Anaplasma FCG. Exposures were made using a 35 mm camera equipped with Kodak Tri-X Pan film. The time of exposure usually varied from two to five minutes, depending upon the brilliance of the field under observation. Prints were made on Kodak Poly-contrast F paper.
B. **Antigenic Relationship to Other Red Blood Cell Parasites.**

Slide specimens of blood containing *Bartonella*, *Eperythrozoon*, *Babesia* and various species of *Plasmodium* were obtained from a number of laboratories. The specimens were stained with normal and anti-*Anaplasma* FCG and examined for specific reaction. Degree of reaction was recorded as negative through 4+, depending upon brilliance of fluorescence.

C. **Electron Microscopy.**

Electron microscopy was used mainly as a method of judging purity or homogeneity in the preparation of experimental antigens. It was also very useful in determining the degree of lysis of red blood cells or damage to AB, size range of particles and occasional observations on the morphology of the AB.

1. **Specimen Preparation.**

Suspensions for examination were prepared by the various methods outlined previously. In most instances, specimens were applied directly to grids which were covered with carbon-coated collodion membranes. When excess salts interfered with observation, the dried specimen area on the grids was washed gently to dissolve the salts and dried under vacuum. Specimens were shadowed with uranium for examination.

2. **Observation and Photography.**

Specimens were observed using a Phillips Model 100 electron microscope. Photographic exposures were made on 35 mm Kodak Panotomic X film and printed on Kodak Polycontrast paper.
RESULTS

A. Complement-fixing Antibodies in Serum Fractions.

Fractionation by continuous-flow paper electrophoresis of serums from normal calves and calves in the acute and convalescent stages of anaplasmosis resulted in the separation of albumin, alpha-, beta-, and gamma-globulins. The average total recovery based on protein analyses after fractionation was 72 per cent, as determined by the biuret method. The relative CF antibody activity in fractions of serums from calves with acute anaplasmosis is shown in Figure 4. In all instances serums contained CF antibodies which were associated with the fractions of alpha- and beta-globulins of lower electrophoretic mobilities and the gamma-globulins of higher mobility. Most of the antibody activity was present in fractions which were identified as beta-globulins. Figure 5 indicates the location of the CF antibody activity in fractions of serums obtained from calves during the convalescent phases of anaplasmosis. Antibody activity in low concentration was found in the beta-globulin fractions of lower mobility. The gamma-globulins of high and intermediate mobilities possessed antibodies in high concentration. These serums from convalescing animals did not contain CF antibodies in fractions migrating with the alpha-globulins, which was in contrast to those detected in acute phase serums. Studies of normal bovine serums did not reveal CF activity in the fractions that were separated; neither did the whole serum show antibody activity. Mean values of the absolute
ELECTROPHORETIC PATTERN OF WHOLE SERUM

RECIPROCAL OF CF TITER OF SERUM FRACTIONS (ORDINATE)

(CF TITER OF WHOLE SERUM 1:640)

Figure 4. Distribution and titers of complement-fixing antibodies in serum fractions of acute phase anti-Anaplasma marginale serum
Figure 5. Distribution and titers of complement-fixing antibodies in serum fractions of convalescent phase anti-\textit{Anaplasma marginale} serum.
concentrations of the individual serum protein fractions for samples obtained during the acute and convalescent stages and for normal serum as determined by paper strip electrophoresis are given in Table II. Serums obtained during the acute phase of infection, as demonstrated by a large number of erythrocytes containing AB, exhibited decreases in absolute values of total serum protein, albumin, gamma-globulin, and total globulins. The concentration of alpha-globulin increased, whereas a significant change in the concentration of beta-globulin was not observed. During the convalescent states, when the erythrocytes that contained AB decreased in number and approached the one per cent level, an increase in the concentration of gamma-globulin occurred and levels of alpha- and beta-globulins decreased below normal values.

B. CF Antigenicity of Stromatal Components.

Stromatal antigen, prepared by lysis and precipitation of pooled infected erythrocytes in carbon dioxide-saturated distilled water, possessed a titer of 1:10 and compared favorably to that of the standard antigen (Anaplasma CF antigen, United States Department of Agriculture). The CF antigenic activity of stromatal components separated by the methods described by Moskowitz and Calvin (1952) is given in Table III. When fractions were titrated with standard positive and negative serums the amount of antigenic activity in the stromin fraction was essentially unchanged from the unfractionated stromata. Titrations of suspensions of elinin derived from the stromin resulted in titers as high as 1:320. However, when a number of standard positive and negative serums were tested, using the elinin
Table II. Means* of absolute concentrations of serum protein fractions in acute and convalescent stages of anaplasmosis and of normal serum.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acute serum</th>
<th>Convalescent serum</th>
<th>Normal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Protein concentration in grams/100 ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gamma-globulin</td>
<td>0.92±.08</td>
<td>2.02±.31</td>
<td>1.54±.19</td>
</tr>
<tr>
<td>beta-globulin</td>
<td>1.43±.11</td>
<td>1.07±.07</td>
<td>1.49±.14</td>
</tr>
<tr>
<td>alpha-globulin</td>
<td>1.62±.27</td>
<td>1.02±.14</td>
<td>1.34±.08</td>
</tr>
<tr>
<td>albumin</td>
<td>2.09±.13</td>
<td>3.12±.24</td>
<td>3.10±.12</td>
</tr>
<tr>
<td>total serum protein</td>
<td>6.06±.19</td>
<td>7.23±.24</td>
<td>7.47±.33</td>
</tr>
</tbody>
</table>

* Mean values for 16 calves in acute stages, 14 calves in convalescent stages, and 18 normal calves.
Table III. Presence of CF antigenic activity in fractions of red blood cell stromata separated by chemical fractionation.

<table>
<thead>
<tr>
<th></th>
<th>Unfractionated stromata</th>
<th>pH 9 supernatant fluid</th>
<th>S protein</th>
<th>Elinin extract</th>
<th>Alcohol: ether extract</th>
<th>Residue (stromatina)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaplasma-infected stromata</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Normal stromata</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>


fraction as the CF antigen, considerable anticomplementary activity in the antigen was demonstrated. Agglutination of the sheep red blood cells also occurred in many of the samples. Upon further fractionation by complete removal of lipids, CF activity was not found in the resulting fractions. Fractions of stromata prepared in a similar manner from normal erythrocytes did not show CF antigenicity.

1. Extraction with Lipid Solvents.

When untreated stromatal antigens were extracted with mixtures of alcohol and ether, and chloroform and methanol, the resulting residues and lipid extracts did not show any CF antigenic activity when tested with anti-Anaplasma serums.

2. Extraction with Fluorocarbon.

High speed homogenization of aqueous stromatal suspensions with Genetron 113 resulted in the separation of three distinct layers. Thick stromatal material was present at the interface of the aqueous layer and the lower Genetron layer. CF tests on the aqueous material revealed it to be highly anticomplementary. Similar results were obtained after repeated extractions of the aqueous layer.

When the sonicates of stromata were extracted with Genetron and the resulting aqueous suspensions tested for CF activity, titers of 1:5 to 1:10 were obtained and anticomplementary activity was not detected. High speed centrifugation of these aqueous suspensions and subsequent testing for CF activity of supernatant fluid and sediment showed that all the active material was present in the sediment. The antigenic activity of the material did not exceed that of standard stromatal antigen in any case. However, the extraction process removed considerable color and undesirable stromatal
material and produced a clearer antigen with no anticomplementary properties.

3. Electrophoresis of Stromata.

Paper electrophoresis of normal and infected stromata, using buffers ranging from pH 2.2 to 9, was found to be of no value when applied to the separation of stromatal components. A band moved out a very short distance at pH 2.2 but no distinct separation of components was evident upon examination of strips dyed with bromphenol blue. Differences were not noted upon comparative analysis of all tracings obtained under these conditions when dyed strips were scanned in the densitometer-integrator. Application of higher current in order to enhance separation did not improve results.

4. Sonic Treatment of Stromatal Antigens.

The results of the effects of sonic vibration at 9 kc/second on Anaplasma CF antigens are given in Figure 6. Exposure of antigens to sonic vibration for periods of up to two minutes consistently resulted in over a four-fold increase in the CF antigenic titer. After five minutes of treatment the antigenic activity progressively decreased to slightly lower levels than the original titers. Anticomplementary activity was not observed in the treated material.

C. CF Antigen Produced by Sonic Treatment.

Suspensions of washed Anaplasma-infected red blood cells were disintegrated by sonic vibration for five minutes followed by removal of residual intact cells and a majority of the hemoglobin by differential centrifugation. This procedure resulted in a final CF antigen preparation with highly desirable characteristics. These
Figure 6. Effect of sonic vibration on the titer of complement-fixing stromatal antigen prepared from Anaplasma-infected erythrocytes.
characteristics manifested themselves as high CF antigenic titer, absence of excessive color due to hemoglobin, and the lack of anti-complementary properties in the dilutions used for testing. Titers of CF antigens prepared in this manner ran as much as 25 times higher than that of standard stromal antigens when tested according to the protocol outlined by the United States Department of Agriculture. When the antigens were tested with standard anti-\textit{Anaplasma} and normal serums, they were found to be highly specific. Antigenic material was not removed by washing the antigen in buffer to remove hemoglobin since the supernatant fluid from the high speed sedimentation processes did not possess CF activity.

1. \textit{Filtration Studies}.

Antigenic activity of serial filtrates of the \textit{Anaplasma} CF antigens described above appear in Table IV. These results indicated the antigenic substance to be of relatively large particle size. The antigenically-active material passed through filters with apparent pore diameters of 650 \textmu m and greater, but material passing through filters of 450 \textmu m failed to show antigenic activity. Initial filtrations resulted in more homogeneous suspensions of the material but, as filters with progressively smaller pore diameters were used, the suspensions became clearer. The filtrate which passed through the 450 \textmu m filter was clear and colorless.

2. \textit{Absorption}.

Normal and anti-\textit{Anaplasma} serums were incubated with the antigen produced from the sonicate of \textit{Anaplasma}-infected erythrocytes to allow absorption to occur. The mixtures were subjected to paper strip electrophoresis and strips were examined for evidence of
Table IV. CF antigenic activity of filtrates* of CF antigen prepared from sonicates of *Anaplasma*-infected erythrocytes.

<table>
<thead>
<tr>
<th>Antigen ml</th>
<th>Unfiltered control</th>
<th>5000</th>
<th>3000</th>
<th>1200</th>
<th>650</th>
<th>450</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.012</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.025</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.050</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>0.100</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>0.150</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>0.200</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>0.250</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>0.400</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>0.500</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

* Passed through Millipore filters.

Negative (-) through 4 indicates degree of complement fixation.
absorption as an alteration in the serum protein pattern of the absorbed serum. Quantitative or qualitative alterations were not evident in the serum protein profiles after strips were dyed with bromphenol blue and scanned with the densitometer-integrator.

3. Antigenicity and Anaplasma Body Count.

Similar antigens were prepared from the sonicates of Anaplasma-infected erythrocytes obtained from carrier animals and animals in the acute stages of the disease with 6.5 to 90 per cent of the erythrocytes containing AB. The CF antigenicity increased as the number of erythrocytes containing AB increased. Table V shows the relative mean CF titers of antigens prepared from equal quantities of packed Anaplasma-infected red blood cells in relation to the percentage of cells containing AB. In the course of this investigation similar observations, which support these results, were made after preparing numerous other experimental CF antigens.

4. Microscopic Examination.

Examination of antigens produced in this manner by electron microscopy and after staining with Giemsa revealed only amorphous stromatal material. Figure 7 is an electron micrograph of a uranium shadowed preparation of the antigen suspension. Concentration of AB was not apparent.

5. Biochemical Characteristics.

A number of qualitative biochemical tests were made on antigens produced from sonicates of Anaplasma-infected red blood cells. The results of tests for protein, carbohydrate, lipid, cholesterol and cystine are given in Table VI. Quantitative protein determinations
Table V. Mean relative CF activity of antigens prepared from the sonicates of erythrocytes with various AB counts.

<table>
<thead>
<tr>
<th>Antigen ml</th>
<th>Normal</th>
<th>Carrier*</th>
<th>6.5</th>
<th>36.5</th>
<th>45</th>
<th>65</th>
<th>75 &amp; 90**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.012</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>0.025</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.050</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.150</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.200</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.250</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.400</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
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<td>4</td>
</tr>
<tr>
<td>0.500</td>
<td>-</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

* Less than one per cent of erythrocytes containing AB.

** Pooled.
Figure 7. Electron micrograph of Anaplasma CF antigen prepared from the sonicate of infected red blood cells. Uranium shadowed.
5500X.
Table VI. Results of biochemical tests on CF antigen prepared from the sonicates of *Anaplasma*-infected erythrocytes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein*</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>-</td>
</tr>
<tr>
<td>Lipid**</td>
<td>+</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>+</td>
</tr>
<tr>
<td>Cystine</td>
<td>-</td>
</tr>
</tbody>
</table>

* Protein concentration = 1.98 per cent as measured by biuret test.

** Phosphatide.
were also made by the biuret method. Phosphatide was the only lipid detected by thin-layer chromatography.

6. **Effects of Enzyme Treatment.**

The antigen was treated with various enzymes and titrated to determine the effects of enzymatic action on the antigenic titer. The results of these experiments appear in Table VII. Only two of the enzymes had apparent destructive effects upon the CF activity of the antigen. The trypsin-treated sample showed almost a complete loss in antigenic activity, and a significant reduction in titer resulted in the lipase-treated antigen. Considerable anticomplementary activity was exhibited by the samples treated with lipase and taka-diastase, although samples exposed to phospholipase A and the nucleases showed a small degree of anticomplementary activity at lower antigen dilutions.

7. **Effects of Temperature.**

Samples of the antigen were treated at 60°C for two hours, at 25°C for 12 hours and repeatedly frozen and thawed. No changes in CF antigenic titer resulted from these treatments.

D. **CF Antigen Prepared by Ribosome Method.**

When the method of preparing ribosomes from reticulocytes by differential centrifugation by the process shown in Figure 2 was applied to Anaplasma-infected erythrocytes, the final sediment produced was found to possess CF antigenicity. Antigenic titers were only as high as 1:10; however, the final material was practically clear when suspended in the CF buffer and possessed only a slight yellow color. Microscopic examination of Giemsa-stained preparations, and by
Table VII. Effects of enzymes on *Anaplasma* CF antigen.

<table>
<thead>
<tr>
<th>Antigen ml</th>
<th>Serum</th>
<th>Untreated Antigen</th>
<th>Trypsin</th>
<th>Taka-diastase</th>
<th>Phospho-lipase A</th>
<th>Lipase</th>
<th>DNAase</th>
<th>RNAase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>+</td>
<td>4</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Negative (-) through 4 indicates degree of complement fixation.
dark-field showed a concentration of *Anaplasma* bodies and a considerable amount of fine stromatal material.

**E. Purification of Anaplasma Bodies.**

Preparations of highly concentrated and semipurified AB were prepared by differential centrifugation after the AB were freed by sonic disintegration of the red blood cells, as outlined in Figure 3. A certain amount of contamination was evident which consisted of fragments of red blood cell stromata. This was revealed by electron microscopic examination of shadowed preparations of suspensions of the material as shown in Figure 8. The final supernatant fluid contained almost all of the AB while the sediment consisted of stromatal material and few AB. Nucleic acid analyses were made on all fractions, to serve possibly as an indicator of the presence of AB. Table VIII shows the data obtained when the two final fractions, prepared from *Anaplasma*-infected erythrocytes, were compared for the presence of AB and CF antigenicity and for nucleic acid content.

When erythrocytes from normal calves were treated in a similar manner, nucleic acids, CF activity or structures resembling AB did not appear in any fraction.

When the method of Ristic (1962) was employed for the purification of AB, fewer large particles of red blood cell stromata were found in the supernatant fluid containing the AB, although considerable fine stromatal material was present. Analyses of sediments and concentrates of AB which were present in the supernatant fluids indicated that CF activity, AB content and concentrations of nucleic acids were approximately the same.
Figure 8. Electron micrograph of suspension of concentrated AB. Uranium shadowed. 10,000X.
Table VIII. Comparison of final supernatant fluid and sediment of AB purification process.

<table>
<thead>
<tr>
<th></th>
<th>Presence of AB</th>
<th>CF antigenicity</th>
<th>RNA</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant fluid</td>
<td>++++</td>
<td>+</td>
<td>-</td>
<td>20μg/ml</td>
</tr>
<tr>
<td>Sediment</td>
<td>+</td>
<td>++++</td>
<td>400 μg/ml 600μg/ml</td>
<td></td>
</tr>
</tbody>
</table>
The concentrated AB were subjected to a number of qualitative tests for the presence of various biochemical constituents and the results appear in Table IX.

Calves were inoculated with the purified AB and the final sediment, which were prepared by this method and with ribonucleic acid extracted from the AB with phenol. Serum samples were tested for 30 days beginning on the day after inoculation. CF antibodies were detected beginning on the 9th day and persisted until the 13th day after inoculation in the calf receiving the final sediment. The purified AB preparation and the ribonucleic acid did not induce a detectable CF antibody response. Infections did not occur in any of the calves.

F. Plasma Sediment.

The sediment of plasma centrifuged at 39,000 x g in a continuous-flow centrifuge did not possess CF antigenicity when tested with standard anti-Anaplasma serum. Microscopic examination of Giemsa-stained preparations, however, showed numerous AB-like structures.

G. Density Gradient.

Sonicates of Anaplasma-infected erythrocytes were layered on gradient columns prepared with buffered saline solutions of sucrose ranging from 300 to 600 mg/ml of sucrose. The gradient columns were centrifuged at 50,000 x g for one hour in a swinging bucket rotor. A schematic representation of a typical sedimentation experiment is shown in Figure 9. Layers were removed in descending order and designated one through six. Layer one contained the majority of the hemoglobin fraction, while layer two contained low density material which
Table IX. Results of biochemical tests on AB concentrated by differential centrifugation of the sonicate of infected red blood cells.

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>-</td>
</tr>
<tr>
<td>Lipid*</td>
<td>+</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>+</td>
</tr>
<tr>
<td>Cystine</td>
<td>-</td>
</tr>
</tbody>
</table>

* Phosphatide.
Figure 9. Distribution of sonicate of *Anaplasma marginale*-infected erythrocytes after density gradient sedimentation in sucrose.
was amorphous in nature as determined by examination of stained preparations. Particulate material, such as AB or cellular debris, was not observed in layers one, three, and four. Layer five, a very compact layer possessing the highest CF titer, was composed of light-brown colored material. When smears of this layer were stained and examined by light microscopy, they were found to contain very high numbers of marginal bodies and considerable amorphous acidophilic- and basophilic-staining material. Examination of this material by electron microscopy confirmed the fact that the layer contained large amounts of amorphous material and marginal bodies. Layer six contained a pellet which was found to consist of acidophilic-staining stromatal material. The layer did not contain CF activity. The distribution of CF antigenic activity in the gradient layers is indicated (Figure 10). The fraction showing the highest antigenic activity (layer five) was relatively free of hemoglobin and, when prepared in the proper dilution for the CF test, was only slightly opaque as compared to standard stromatal antigens. Anticomplementary activity was not encountered. Recycling of layer five resulted in a preparation containing AB in a higher state of purity, but an increase in CF activity was not observed. Sucrose had no apparent effect on the CF reaction.

In order to determine whether the material used for preparing gradients had any effect on the infectivity and in vivo antigenicity, susceptible animals were inoculated with mixtures of the sonicates of infected erythrocytes and solutions of glucose and polyvinylpyrrolidone. Animals were also inoculated with the layer containing AB which was prepared by repeated centrifugation
Figure 10. Distribution of *Anaplasma marginale* CF antigenic activity in fractions of sonicate of infected erythrocytes separated by density gradient in sucrose.
in sucrose gradient columns. Infection was produced in two out of three calves receiving polyvinylpyrrolidone and glucose-treated inoculums. Animals inoculated with purified AB failed to show infection. Serums from three of the animals receiving the treated sonicates were checked and found to contain Anaplasma CF antibodies.

H. Immunologic and Structural Relationships of Anaplasma Bodies.

1. Fluorescent Antibody Studies.

Normal and anti-Anaplasma FCG were prepared from standard serums and tested against Anaplasma-infected and normal blood. Figure 11 presents a photomicrograph of a typical anti-Anaplasma FCG-stained thin smear of blood from an animal with acute anaplasmosis. In examining various preparations of this nature, areas of diffuse fluorescence were noted adjacent to or surrounding the AB (Figure 11). Such diffusely fluorescing areas were also observed in other cells which did not contain AB.

FCG-stained preparations of sonicates of infected red blood cells were also studied. Examination of the same specimen areas by regular dark-field revealed that many AB which remained intact failed to fluoresce after sonic treatment. Figure 12 shows a photomicrograph of such an FCG-stained preparation using ultra-violet illumination, and Figure 13 shows the same field using regular dark-field with tungsten illumination. It may be seen by comparing the two photomicrographs that a greater number of AB are visible under tungsten illumination (Figure 13) than under ultra-violet illumination (Figure 12). Table X summarizes the results of observations made on various FCG-stained preparations.
Figure 11. Anti-Anaplasma FCG-stained thin-smear of blood from an animal with acute anaplasmosis. Arrows indicate AB and areas of diffuse fluorescence within erythrocytes. 1700X
Figure 12. Anti-Anaplasma FCG-stained thin-smear of Anaplasma-infected erythrocytes after treatment with sonic vibration for one minute. Photographed with ultraviolet illumination. 1700X.
Figure 13. Anti-Anaplasma FCG-stained thin-smear of Anaplasma-infected erythrocytes after treatment with sonic vibration for one minute. Identical field as shown in Figure 12 but with tungsten illumination. 1700X.
Table X. Reactions observed when various preparations were stained with anti-\textit{Anaplasma} FCG.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Normal erythrocytes</td>
<td>No reaction</td>
</tr>
<tr>
<td>b. \textit{Anaplasma}-infected erythrocytes</td>
<td>Fluorescent AB</td>
</tr>
<tr>
<td>c. Sonicate of \textit{Anaplasma}-infected erythrocytes</td>
<td>Fluorescence of some AB but not all</td>
</tr>
<tr>
<td>d. Purified AB</td>
<td>Very few fluorescing AB</td>
</tr>
<tr>
<td>e. \textit{Anaplasma} stromatal CF antigen</td>
<td>Fluorescence of amorphous particles but few AB</td>
</tr>
<tr>
<td>f. \textit{Anaplasma} CF antigen prepared from infected cells</td>
<td>Fluorescence of amorphous particles. No AB observed.</td>
</tr>
</tbody>
</table>
Due to similarities in development between *Anaplasma* and *Plasmodium* (Cane *et al.*, 1963) the possible antigenic relationships of *A. marginale* to various red blood cell parasites were studied by fluorescent antibody methods. Unfixed, unstained blood smears of Bartonella, Babesia, Eperythrozoon and a variety of *Plasmodium* species were obtained from various investigators throughout the country. Normal and anti-"Anaplasma" FCG were applied to smears, and the results of microscopic examinations appear in Table XI. Strong fluorescent reactions were obtained when *P. inui*, *P. cynomolgi* and *P. gallinaceum* were stained with anti-"Anaplasma" FCG. Reactions were also obtained when *P. gallinaceum* and *P. cynomolgi* were stained with normal FCG, although to a lesser extent with *P. cynomolgi*. Figures 14, 15 and 16 contain photomicrographs of blood infected with these species of *Plasmodium*, stained with normal and anti-"Anaplasma" FCG. Babesia, Bartonella, Eperythrozoon and *Plasmodium* species *brasiliannum*, *circumflexum*, coatneyi, elongatum, hexamerium, lophurae and vaughani did not fluoresce when stained with normal and anti-"Anaplasma" FCG. A slight reaction was observed when *P. berghei* was stained with normal and anti-"Anaplasma" FCG.

2. **Electron Microscopy.**

Electron microscopy was employed primarily as a means of judging the purity and homogeneity of various antigen preparations. Furthermore, in order to recognize free AB when observed by electron microscopy, partially lysed preparations of Anaplasma-infected erythrocytes were studied. Figure 17 shows such a preparation with a free AB adjacent to an intact erythrocyte ghost which contains an AB. The free AB appears to correspond with the "doughnut" forms.
Table XI. Reaction* of normal and anti-Anaplasma FCG with various other red blood cell parasites.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Normal FCG</th>
<th>Anti-Anaplasma FCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmodium berghei</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>&quot; brasiliannum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; cathemerium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; circumflexum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; coatneyi</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; cynomolgi</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>&quot; elongatum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; gallinaceum</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>&quot; hexamerium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; inui</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>&quot; lophurae</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; vaughani</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Babesia canis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bartonella sp.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eperythrozoon wenyoni</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Positive reactions rated + through ++++ depending upon brilliance in relation to controls.
Figure 14. Thin-smears of blood infected with *P. inui*. Arrows show parasite within erythrocyte membrane. 1700X.

Upper. Stained with anti-*Anaplasma* FCG.

Lower. Stained with normal FCG.
Figure 15. Thin-smears of blood infected with *P. cynomolgi*. Arrows show parasite within erythrocyte membrane. 1700X.

Upper. Stained with anti-*Anaplasma* FCG.

Lower. Stained with normal FCG.
Figure 16. Thin-smears of blood infected with P. gallinaceum. Arrow shows parasite within erythrocyte membrane. 1700X.

Upper. Stained with anti-Anaplasma FCG.

Lower. Stained with normal FCG.
Figure 17. Electron micrograph of free AB adjacent to erythrocyte ghost containing AB. Uranium shadowed. 60,000X.
which are frequently observed. An electron micrograph of a similar structure at a much higher magnification is shown in Figure 18.

Occasionally, erythrocytes were observed which contained numerous small structures (Figure 19) presumed to represent the elementary bodies which make up the AB or marginal body (Ristic, 1960c).
Figure 18. Electron micrograph of free "doughnut" AB. Uranium shadowed. 135,000X.
Figure 19. Electron micrograph of erythrocyte ghost containing *Anaplasma* elementary bodies. Uranium shadowed. 40,000X.
A. Serum Protein Changes and CF Antibody Content in Serum Fractions.

Results described in this treatise confirm earlier observations (Dimopoullos et al., 1960) on changes which occur in the serum proteins of calves infected with *A. marginale*. The present studies also give evidence in favor of a previous suggestion (Dimopoullos et al., 1960) concerning a shift in mobility of the CF antibodies which are resident in the serum globulins. The shift coincides with changes in the concentrations of serum globulins in the acute and convalescent phases of anaplasmosis.

Similar changes in the electrophoretic mobility of other antibodies which are associated with various serum protein fractions have been reported. Such a shift was noted by van der Scheer et al. (1940) in a study of anti-pneumococcal sera. The antibody produced during early stages of immunization possessed a mobility between the beta- and gamma-globulins while practically all of that formed later was found associated with the gamma-globulin. More recent work by Brown and Graves (1959) and Brown (1960) showed the same direction of shift in precipitating antibodies of foot-and-mouth disease. Seven days after infection the precipitins appeared in the beta-globulin region and after 14 days precipitins were found in the gamma-globulin region. It was also shown that the neutralizing and precipitating antibody activities of a given serum were in one fraction. Shifts in the opposite direction have been described by Zinneman et al.
(1959) and Glenchur et al. (1961) in experiments dealing with blocking antibodies to Brucella in human and rabbit serums. Over a period of two months the antibody activity in the gamma-globulin became associated with the beta-globulin.

Even though the present finding cannot be compared strictly with the others mentioned above, due to the study of different antibodies, the similarity of changes in antibody location within the serum proteins cannot be overlooked. It is evident that changes occur in the location of CF antibody activity among the serum protein fractions in anaplasmosis. Specifically, there is a disappearance of the CF activity associated with the alpha-globulin of lower electrophoretic mobility in the convalescent phases as compared to the activity present in the alpha-globulin in the acute phase. There is no appreciable change in the CF activity of the beta-globulin during the disease except that lower CF titers are observed in this fraction during the convalescent stages. The gamma-globulin fractions appear to change most drastically. In the acute phases only the fraction of highest mobility shows CF activity, whereas during the convalescent stages the CF activity of the gamma-globulin has become associated with the fractions of higher and intermediate mobilities.

The changes mentioned above, that is the apparent shift of CF activity toward globulins of lower mobilities, may be due to inherent structural differences of the CF antibodies present in the serum fractions which develop during the disease, specifically, the period between the acute and convalescent phases. These changes could account for a difference in net charge as measured by these electrophoretic methods and would be evidenced by changes in the location
of the CF activity due to the different electrophoretic mobilities.

There is a need for further work to determine whether other antibodies are produced in anaplasmosis which could be of diagnostic significance. Immunoelectrophoretic studies of serums from animals with anaplasmosis, using antigens prepared from various fractions of infected erythrocytes, would be of aid in elucidating other immunological manifestations which may be involved in the disease.

B. Anaplasma CF Antigen.

Fractionation of stromata prepared from Anaplasma-infected erythrocytes showed that the CF antigenic substance was present in the fractions containing stromin and elinin. The retention of all CF antigenic activity by the fraction containing stromin is not particularly significant except that it demonstrates the relative stability of the CF antigen in an alkaline solution. In addition, stromatal CF antigens may be cleared of almost all of the hemoglobin by this treatment so that color no longer interferes with the determination of the degree of hemolysis in the CF test. As pointed out earlier in the review of the development of the Anaplasma CF antigen this has been one of the major problems with stromatal preparations of the type presently used for routine diagnosis. There is an apparent loss of specificity upon extraction of ether-soluble lipids to produce the fraction containing elinin. In addition, the loss of CF activity which results when all lipids are removed by extraction with alcohol:ether or methanol:chloroform indicates that the antigen has an active lipid moiety. Neither the lipid extracts
nor the protein residue retained CF antigenicity. These characteristics present favorable evidence that the antigen is lipoprotein in nature. The possibility of denaturation of the antigenic substance by these procedures has been considered. However, purification of blood group antigens by the same methods has been successful (Moskowitz et al., 1950).

Fluorocarbons are used for isolation of virus particles in order to have greater control over the specific gravity and viscosity of the isolating medium (Gessler et al., 1956a). In virus isolation methods such solvents allow separation of the nucleoproteins of viruses from nonviral proteins and lipids. According to Gessler et al. (1956b) the principle involved is a preferential wetting or attraction of the surface of the viral particle by water while non-viral protein particles and lipids are preferentially attracted by the fluorocarbon solvents. This method was applied to homogenates of Anaplasma-infected erythrocyte stromata as a possible means of obtaining CF antigenic material of high purity and activity. The factors responsible for failure to obtain fractions which were antigenically-active when stromata were homogenized and extracted with Genetron 113 are not clear. However, similar treatment of sonicates of the stromata resulted in an aqueous layer which contained CF-active material. This is probably due to inadequate homogenization or lack of disintegration of erythrocyte stromata in the former case and as Gessler et al. (1956b) have pointed out, a very thorough mincing and homogenization of tissue is important. Sonication, therefore, apparently liberated more of the antigenic substance in the form of small particles which remained in the aqueous layer of
lower density than the Genetron 113. The absence of anticomplementary activity in the aqueous layer was probably accounted for by the removal of this activity in the dense stromatal material separated at the interface by the centrifugation process. A certain amount of lipid extraction by the fluorocarbon solvent probably occurred and antigenic activity may have been decreased by the removal of lipid. For example, Halonen et al. (1958) found that fluorocarbons reduced the specific reaction of ECHO CF antigens. The addition of serum aided in preventing loss of specific activity. Another possible explanation for low titers in the aqueous layer is that CF activity is not completely extracted from the stromatal material even by multiple treatments. Such experiments might prove helpful in dealing with the purification and concentration of Anaplasma antigens by these methods.

Sonic vibration has been used to liberate the contents of erythrocytes in further efforts to obtain a superior Anaplasma CF antigen. Since the organism has been shown to be somewhat resistant to sonic vibration (Bedell and Dimopoullos, 1963) it was of interest to determine the effects of sonic treatment on the CF antigenic activity of stromatal antigens. Exposure of antigens to sonic vibration for periods of up to two minutes resulted in a fourfold increase in CF antigenic titer (Figure 6). These results indicate that dispersion of the stromatal material or disruption of the AB may increase the number of active antigenic sites available for reaction with antibody. Further exposure of the antigen probably destroys configurations on the molecules of antigen which are responsible for the CF reaction. These results point out that controlled
sonic treatment may be of significance in the preparation of antigens by this method.

Disintegration of Anaplasma-infected erythrocytes by sonic vibration followed by the removal of residual intact cells and hemoglobin by differential centrifugation resulted in a preparation with high CF antigenic activity. Titers ordinarily obtained with standard stromatal antigens range from 1:10 to 1:15 while the antigens prepared by this method possess titers as high as 1:250 when tested with identical standard anti-Anaplasma serums. Moreover, the complete absence of red color due to hemoglobin and lack of significant anti-complementary activity are very encouraging. The antigen preparation was found to be stable after freezing and thawing and was not affected by heating for two hours at 60 C. Preliminary testing of numerous known anti-Anaplasma and normal serums indicates that the antigen preparation has a high degree of specificity. When serums of known antibody titers were tested with standard stromatal and experimental antigens the titers were found to deviate no more than one dilution, if any. Such an antigen would lend itself favorably to use in a micro-CF test for anaplasmosis.

Density gradient sedimentation of sonically-disintegrated preparations of Anaplasma-infected erythrocytes was also useful in fractionation of CF antigens associated with the infected red blood cells. Antigens obtained by this method contained very little color since most of the hemoglobin did not migrate with the CF-active fractions during the sedimentation process. The fractionation resulted in the preparation of an antigen of higher CF titer than standard stromatal antigens but lower than those produced by
differential centrifugation of sonicates as previously described. The lower titers were encountered in the fractions obtained by the density-gradient method because CF activity was contained in more than one fraction. Much of the inactive cellular debris separated at a different rate than the active portion. This probably accounted for the lack of anticomplementary activity in the antigenically-active fractions. The problem of interpretation of the test also becomes greatly diminished since the preparation, when diluted to the proper concentration for CF testing, is only slightly turbid in contrast to whole stromatal antigens. These results show that density-gradient sedimentation can be employed to prepare Anaplasma antigens which are specific, relatively pure, and of high titer.

A number of studies were made on the antigen prepared from sonicates of Anaplasma-infected erythrocytes in an effort to determine the nature of the CF antigen and its relationship to the AB. Filtration studies of the experimental antigen showed that the CF antigenic substance passed through filters with pore diameters of 650 μ. However, no activity was found in filtrates which passed through filters having a pore size of 450 μ. These results were rather surprising since Allbritton and Parker (1962) conducted filtration studies which showed that the infective agent passed through filters having average pore diameters of 300 μ. The amount of material required to produce infection might however, be much less than that required for detection by the CF test. The presence of a small number of elementary bodies might induce infection but it is not likely that a minute amount of material would possess sufficient antigenic activity to be detectable by the CF test. This is one
possibility since the size range of the infective agent was estimated to be between 220 and 300 μm (Allbritton and Parker, 1962; Ristic, 1960b). Generally, it is recognized that the CF active substance in viruses is smaller than the infectious particle. However, in the present studies the CF antigenic substance appeared to be composed of relatively large conglomerates of amorphous material.

Another possibility is that the infectious material which passed through the pores of 350 μm diameter may not have possessed any CF antigenicity. There is support for this in the fact that suspensions of concentrated semipurified AB, prepared by differential centrifugation as outlined in Figure 3, possessed very low CF antigenic activity. The CF activity was present in a fraction composed of stromatal material and few AB. This will be discussed further in a forthcoming section.

The activity of antigens prepared from the sonicates of infected erythrocytes increased as the number of erythrocytes containing AB increased. However, this should not imply that the AB, per se, constitutes the CF antigen. To the contrary, antigens prepared by this method contained little or no AB, as revealed by electron microscopy of suspensions of such preparations. Examination of these antigens again revealed only amorphous stromatal material. Jensen (1957) has classified antigens resulting from production of virus into two categories: integral substances important in the structure and function of virus particles and antigenic materials released or extracted from infected cells, and often referred to as soluble antigens. Furthermore, it has been found that the CF antigen
is not identical with the infectious particle in virus diseases such as foot-and-mouth disease (Bachrach, 1950).

Significant observations on the relationship of the CF antigen to the AB were made when studies were conducted on fractions produced in purifying AB. After separation by differential centrifugation of the sonicates of infected erythrocytes, the supernatant fluid which contained relatively pure AB was found to be low in nucleic acids, and in CF antigenic activity. On the other hand, the sediment which constituted by-products of the semi-purified AB and contained large quantities of nucleic acids exhibited high CF antigenic titers. These observations likewise suggest that the CF antigen may not necessarily be associated with the AB, _per se_, but may be some material contained within or a coating around the AB, or possibly associated with the erythrocytic membrane. Moreover, since infection has not been produced with purified AB, there is even some question concerning the infectious ability of the AB, especially in view of their very low nucleic acid content. However, the AB has been generally accepted to be the infectious entity (Foote et al., 1958; Ristic, 1962).

Results obtained (Table X) when various preparations were stained with normal and anti-Anaplasma FCG indicate that only the outer coating of the AB reacts with the anti-Anaplasma FCG and that this coat is extremely fragile when compared with the inner structure of the body. This was particularly evident upon examination of FCG-stained preparations of infected erythrocytes partially disintegrated by sonic vibration. The number of AB reacting with the FCG was significantly decreased after sonic treatment. When concentrated
semi-purified AB were obtained by similar methods, only a few AB reacted with the FCG while the sediment contained large quantities of fragmentary material which reacted specifically with the Anaplasma FCG. A hypothetical scheme is given in Figure 20 which may aid in explaining these observations. The highly active CF antigen prepared from sonicates of infected erythrocytes likewise contained only amorphous fragments of membranous material yet possessed a high degree of specificity for anti-Anaplasma FCG.

In many instances certain erythrocytes containing AB also exhibited areas of diffuse material which reacted with anti-Anaplasma FCG (Figure 11). Although structures of various shapes, such as tails (Madden, 1962), have been shown to be associated with the classical punctate AB, the diffusely fluorescing areas have not previously been observed and no significance has been given to them. This phenomenon might indicate that erythrocyte structures, per se, may be partially responsible for the CF antigen after formation of an auto-antigenic erythrocyte (Ristic, 1961) during the disease. Another possibility is that a portion of the antigenic matrix around the AB was broken away from the central body and dissipated within the erythrocyte, resulting in the diffusely fluorescing areas.

Examination of photomicrographs of intact infected erythrocytes shows that certain AB are visible under tungsten illumination (Figure 13) while they are not observed under ultraviolet illumination (Figure 12). This may indicate that these AB may not have reached a particular stage in their development; that is, complete formation of the body with an outer antigenic coat. Ristic and Watrach (1963) have advanced the hypothesis that a complete developmental
Figure 20. Hypothetical scheme for effects of sonic vibration on AB and reaction of components with FCG. Lines around periphery of structures indicate fluorescence.
cycle of Anaplasma occurs in mature erythrocytes. They proposed that the organism reproduces by binary fission of the initial body and that each body is contained in a double membrane. Although this hypothesis may be correct, it does not explain the presence of AB which do not fluoresce within erythrocytes. Examination of thin-sections of Anaplasma-infected erythrocytes by electron microscopy has shown central dense structures surrounded by a membrane (Foote et al., 1958; Ristic, 1960). The reports presented by these workers are compatible with the present FCG studies. The membrane apparently is associated with a thick matrix substance which surrounds the central bodies (Ristic, 1960c). This matrix material may be seen covering the AB (Figures 17, 18) and may represent the antigenic coat.

Qualitative biochemical tests on the CF antigen prepared from sonicates of Anaplasma-infected erythrocytes resulted in positive tests for protein, phosphatide and cholesterol, while protein concentration was estimated to be 1.98 per cent. These results substantiate earlier observations on the biochemical nature of the antigen revealed by fractionation using lipid solvents. Further support for the lipoprotein nature of the antigen was obtained as a result of studying the effects of various enzymes on the antigen. The only enzymes which had an apparent destructive effect upon the antigenicity of the preparation were trypsin and lipase. It would be interesting to determine the effects of these enzymes on the infectivity of Anaplasma.

The significance of the serologic reactions demonstrated between certain species of Plasmodium and anti-Anaplasma FCG is not clear. Concurrent work (Cane et al., 1963) has indicated a
similarity in the developmental cycle of Anaplasma and Plasmodium. Fluorescent antibody studies of hepatic reticulo-endothelial cells from animals during the incubation period of anaplasmosis showed that many of the cells contained material which was specific for anti-Anaplasma FCG. Subsequent detection of other developmental forms in erythrocytes of the peripheral blood provided additional support (Cane et al., 1963). Insomuch as the immunological relationship suggested by the FCG studies presented herein are concerned, any conclusion must await reciprocal studies using anti-Plasmodium FCG.
SUMMARY AND CONCLUSION

Investigations were conducted to determine the location and activity of CF antibodies in the serum protein fractions of animals in the acute and convalescent stages of anaplasmosis. In acute phase serums CF activity was associated with the alpha-globulin of lower mobility, the beta-globulin of lower mobility, and the gamma-globulin of highest mobility. Similar studies with convalescent serums showed a complete absence of CF activity in the alpha-globulin. Activity in the beta-globulin was distributed similar to that found in acute serums although titers of the individual beta-globulin fractions were diminished. The CF activity in the gamma-globulin during the convalescent stages was associated with fractions of high and intermediate mobilities. It was confirmed that a relationship existed between the concentrations of globulin components and CF antibody titers.

Concomitant studies were conducted to determine the nature of the Anaplasma CF antigen and to determine its relationship to the infected erythrocyte and the organism. CF antigenic activity was found in stromin fractions which were prepared from stromata of these antigens. Lipid extractions were deleterious to antigenic CF activity. The results suggested that the CF antigen is lipoprotein in nature.

Homogenization of aqueous suspensions of stromatal sonicates with Genetron 113 showed that considerable color and undesirable stromatal material can be removed from the stromatal antigen by centrifugation.
Treatment of stromatal antigens by sonic vibration increased the antigenic activity.

A method was developed for the preparation of a highly desirable *Anaplasma* CF antigen. The method involved disintegration of infected erythrocytes by sonic vibration and separation of the antigen by differential centrifugation. Antigens prepared by the method were highly specific, colorless, exhibited no significant anticomplementary activity and possessed high titers. Filtration studies showed that the antigenic substance was of relatively large particle size. Microscopic examination of the antigen preparation showed the presence of amorphous stromatal material and no AB. The results of qualitative biochemical studies supported earlier observations on the lipoprotein nature of the antigen.

AB were freed by sonic disintegration of erythrocytes and concentrated by differential centrifugation of the sonicate. CF antigenic activity and nucleic acid content of the AB were low while a by-product contained large amounts of nucleic acids and high CF antigenic activity and reacted specifically with fluorescent antibody.

Erythrocytes infected with *Anaplasma* were disrupted by sonic vibration, and the sonicate was fractionated by density gradient sedimentation. The CF active antigenic material was concentrated in one fraction.

Data obtained using the CF test and the fluorescent antibody technique suggest that an antigenic matrix coats the AB and may be torn away by physical disruption. This coat appears to be responsible for the CF antigenic activity.
Fluorescent antibody studies using anti-Anaplasma FCG and blood smears containing several species of Plasmodium suggest that common antigens might be present in both genera.
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VITA

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Major Field: Bacteriology

Title of Thesis: Immunologic and Serologic Studies of Anaplasma Marginale

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