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Louisiana State University, Ph.D., 1966
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FATTY ACID ANALYSES OF BOVINE ERYTHROCYTES DURING ANAPLASMOSIS AND EPERYTHROZOONOSIS

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 Microbiology

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ABSTRACT

The fatty acids of erythrocytes from splenectomized calves were analyzed before and after infection with *Anaplasma marginale* and *Eperythrozoon wenyoni*. Alterations in fatty acid compositions were followed by periodic assays of the total lipid extract and of the various classes of lipids.

Lipids were extracted from erythrocytes of 4 splenectomized calves throughout experimental anaplasmosis. Samples from corresponding days were pooled and the methyl esters of fatty acids analyzed by gas-liquid chromatography. The principal fatty acids detected were C 16:0, C 18:0, C 18:1, C 18:2, C 22:U, and C 24:0. Prior to inoculation of the calf, lipids contained approximately 30 percent of C 18:1 as the predominant fatty acid. During the height of parasitemia, almost 40 percent of the total fatty acids were contributed by C 24:0. Furthermore, drastic decreases in concentrations of C 18:0, C 18:2, and C 22:0 occurred during the disease. The ratio of saturated to unsaturated fatty acids increased markedly as a result of the infection, reflecting the increase in concentrations of saturated fatty acids and the decrease in concentrations of unsaturated fatty acids.

Lipids from three additional calves infected with *A. marginale* were fractionated into sterol esters, triglycerides, free fatty acids, sterols, and phospholipids by thin-layer chromatography. Fatty acids

in each fraction except the sterols were analyzed by gas-liquid chromatography and data from corresponding days were averaged. In most cases, changes in the fatty acid concentrations of the total lipid fraction were a reflection of the changes in the phospholipid fraction. The C 18 unsaturated fatty acids and C 22:0 showed varied increases in response to the infection. Most of the fatty acids of chain lengths shorter than C 18:0 exhibited a tendency to decrease in concentration.

Dual infections of anaplasmosis and eperythrozoonosis were studied in 4 calves. Fatty acid analyses were conducted on erythrocytic lipids throughout the first acute phase of concurrent infections. Minimal alterations occurred in fatty acid compositions, chiefly in C 20:4, C 22:0, C 22:U, and C 24:0.

Fatty acids were determined in the classes of erythrocytic lipids from calves with experimental eperythrozoonosis. The longer chain fatty acids tended to increase in concentrations, while the fatty acids of carbon chain lengths shorter than 18 showed minor variations with slight decreases in concentrations.

Marginal bodies were isolated from *Anaplasma*-infected erythrocytes, purified by differential centrifugation, and analyzed for fatty acid composition. The total lipid extract and the lipid fractions of sterol esters, triglycerides, free fatty acids, and phospholipids were chromatographed to quantitate the fatty acids.

Unfractionated lipid contained C 16 and C 18 accounting for 85 percent of the total fatty acids, with C 18:1 accounting for 45 percent. The fatty acids in the sterol ester fraction were rather uniformly distributed, but the other three fractions contained higher concentrations of the C 16 and C 18 series and were similar to the values found in the total lipid. The phospholipid fraction contained approximately 48 percent of C 18:1.

INTRODUCTION

The red blood cell has one vital reason for existence--to carry oxygen from lungs to all cells of the body and to return with carbon dioxide (Bishop and Surgenor, 1964). All blood cells originate from undifferentiated mesenchymal or stem cells with nucleus, mitochondria, and microsomes which give them the ability to perform almost any metabolic reaction. The maturing mammalian erythrocyte loses its nucleus and subcellular particles, yet retains an impressive array of enzymes, proteins, carbohydrates, lipids, anions, and cations, many of which have been demonstrated to be in active metabolic flux (Harris, 1963). Like all cell membranes, that of the red blood cell consists essentially of protein and lipid, the two materials being closely associated as lipoprotein complexes (Prankerd, 1961).

When the efficiency of the blood as an oxygen carrier is reduced below normal, as a result of a deficiency of erythrocytes or of hemoglobin or both, the animal is said to have an anemia. Anemia is not a disease *per se*, but rather a state of the blood resulting from inadequate production of erythrocytes or rapid destruction of erythrocytes (Schalm, 1965). Data reported in this dissertation concern two types of anemia in calves caused by rapid destruction of erythrocytes. The anemias were produced by the infectious agents, *Anaplasma marginale* and *Eperythrozoon wenyoni*.

Anaplasma infection in cattle is characterized by the presence of tiny spherical bodies in the cytoplasm of the erythrocytes near the periphery of the cell. The disease causes weakness, pallor of mucosae, accelerated respiration, jaundice, decreased red cell count, and decreased hemoglobin. Death occurs in many cases and the animals which survive become carriers for life.

Eperythrozoonosis in cattle is a disease which is primarily recognized as a problem in splenectomized animals. The intact animal presents no clinical manifestations, whereas, the asplenic animal suffers from general debilitation, mild fever, and severe anemia. Eperythrozoon are seen in blood smears stained with Giemsa's stain as tiny pleomorphic structures which are within the erythrocytes, lying on their surface, or free in the plasma (Smith and Jones, 1957). Death may occur in splenectomized animals, but animals more frequently recover and are prone to have recrudescenses.

Both of the described diseases have parasitic forms associated with circulating erythrocytes and both cause moderate to severe anemia in the host. In an effort to elucidate the pathogenesis of the diseases on a molecular basis, fatty acids in infected red blood cells were assayed qualitatively and quantitatively.

Increasing interest has been shown in the mechanisms of various anemias. Lipids of various kinds are an integral part of the membrane and an understanding of their role in the cell largely depends on

adequate quantitative chemical data. Particular attention has been given to the phospholipids since they serve as major structural components of the erythrocyte (Diziemian, 1939; Ponder, 1949; van Deenen, 1965).

Anaplasmosis reportedly causes a reduction in concentrations of phospholipids in infected red blood cells (Dimopoullos and Bedell, 1962; Schrader and Dimopoullos, 1963). Erythrocytes from *Anaplasma*-infected calves also have increased osmotic fragilities (Dimopoullos and Bedell, 1962), increased electrophoretic mobilities (Dommert and Dimopoullos, 1966), and apparent increased concentrations of sialic acids (Dommert, 1963) indicating that alterations in the erythrocytic membranes do occur. The studies in this dissertation were designed to determine the changes in fatty acid composition of the erythrocytic lipids associated with *Anaplasma* and *Eperythrozoon* infection in calves.

SELECTED LITERATURE

A. Anaplasmosis

Anaplasmosis was first described in the United States by Smith and Kilborne (1893) while investigating Texas fever. They recognized "coccus-like bodies" in erythrocytes, but they regarded them as developmental forms of *Babesia bigemina*. Theiler (1907) in South Africa first recognized the individuality of the organism, and he later named it *Anaplasma marginale*. The etiologic agent has been classified as a rickettsial organism in *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957) although subsequent research (Espana *et al*., 1959; Cane *et al*., 1963) has made the classification questionable.

Theiler considered *A. marginale* to be a protozoan organism and several investigators still support this view (Espana *et al*., 1959; Cane *et al*., 1963; Dommert *et al*., 1965). Other workers advocate a viral classification (Foote, 1954; Foote *et al*., 1958) or a less definite classification as between viruses and rickettsiae (Ristic, 1960).

The disease has been studied in various parts of the world for several decades and much knowledge has been acquired about its epizootology, transmission, etiology, pathogenesis, and diagnosis (Ristic, 1960). Important gaps remain, however, such as the

classification and life cycle of the etiologic agent, the mechanism by which the organism produces anemia, and development of a suitable vaccine or effective treatment for acutely ill cattle. Research on this disease has received impetus from the great economic losses which it causes by deaths and losses in production of beef and milk. The U. S. Livestock Sanitary Association has estimated the loss at $35 million in an average year.

Since anaplasmosis is characterized by a severe anemia and the presence of marginal inclusions in circulating erythrocytes, there has been increasing interest in studying the physico-chemical changes which occur in the parasitized red blood cells. Histochemical techniques indicated that both deoxyribonucleic and ribonucleic acids were present in the marginal inclusions of infected erythrocytes (Penha, 1930; Moulton and Christensen, 1955; Gainer, 1961). Quantitative chemical analyses of Anaplasma-infected stromata demonstrated that parasitized cells have decreased concentrations of phospholipids (Dimopoullos and Bedell, 1962, 1965; Schrader and Dimopoullos, 1963), with increased amounts of deoxyribonucleic acid, ribonucleic acid (Gough, 1963), and sialic acid (Dommert, 1963; Dommert et al., 1963).

Various physical properties of infected blood cells have also been investigated. Such cells reportedly are more susceptible to osmotic lysis (Dimopoullos and Bedell, 1962); they have enhanced

electrophoretic mobilities (Dommert, 1963; Dommert and Dimopoullos, 1966; Dommert et al., 1965); and their stromata flocculate more slowly than uninfected stromata (Dimopoullos and Bedell, 1961, 1965; Dimopoullos, 1964). Bedell and Dimopoullos (1962, 1963, 1965) studied the effects of temperature and sonic energy on the infectivity and virulence of A. marginale in whole blood preparations. They found that as the time of exposure to sonic energy or a particular temperature increased, the incubation period of the disease also increased; however, treatment of infected blood with sonic energy resulted in higher peak counts of infected cells in inoculated calves. Similar increases in incubation periods were noted when cobalt-60 was used to irradiate whole infected blood (Gough and Dimopoullos, 1965) and infectious marginal body preparations (Wallace and Dimopoullos, 1965b).

Following the report that marginal bodies could be isolated without destroying their infectivity (Wallace and Dimopoullos, 1965b), several properties of the partially-purified agent have been studied. Isolated marginal bodies were found to have approximately the same resistance to ionizing radiation as whole, infected blood (Wallace and Dimopoullos, 1965b). Analyses of the partially-purified body preparations for enzyme activity have revealed the presence of catalase (Wallace and Dimopoullos, 1965a), cholinesterase (Wallace, 1966), and lactic dehydrogenase (Darrè, 1966). Earlier reports on the nature of the complement-fixing antigen in anaplasmosis

indicated that the antigen was lipoprotein in nature (Rogers et al., 1964; Rogers and Dimopoullos, 1965).

B. Eperythrozoonosis

Eperythrozoonosis in Louisiana cattle has been described as inapparent or unrecognized in nonsplenectomized animals. It occurs most often in calves after splenectomy (Dikmans, 1933; Lotze and Yiengst, 1941; Jensen, 1943; Foote et al., 1957), yet Crocker and Sutter (1954) described a serious outbreak in 35 intact cows shipped from Texas to Kentucky.

The disease in cattle was first recognized by Adler and Ellenbogen (1934) who described a minute parasite of the erythrocytes of cattle and named it *Eperythrozoon wenyoni*. The organism has been observed in the blood of cattle in Palestine (Adler and Ellenbogen, 1934), South Africa (Neitz, 1940), France (Donatien and Lestoquard, 1937), and the United States (Dykstra et al., 1948).

When stained with Giemsa stain, the parasites appear pinkish-purple in color and are ring-like, ovoid, comma, rod, dumbbell, or tennis racket-shaped. They range in size from 0.3 to 1.5 microns in diameter (Henning, 1956). The organism has been variously described as occurring in the erythrocyte (Adler and Ellenbogen, 1934), on the surface of the erythrocyte (Nieschulz, 1938), both in and on the surface of the cells (Henning, 1956), and in the plasma (Hoyte, 1962). A new species name, *E. teganodes*, was proposed by Hoyte

(1962) for the Eperythrozoon which was found in the plasma but not in or on the erythrocytes of bovine blood. He also suggested that some highly pleomorphic species of Eperythrozoon may prove to be a mixture of two or more species.

The incubation period of the infection varies from 16 to 22 days and parasites are demonstrable for periods varying from 1 to 8 days. A latent infection with E. wenyoni in cattle can be activated by heavy tick infestation, by anaplasmosis, and by a severe infection of Theileria mutans (Henning, 1956). Severe clinical symptoms of fever, inappetence, depression, acute muscular weakness, icterus, and anemia were observed only in splenectomized cattle. Intact animals exhibited only slight anemia, but no febrile reaction, definite clinical symptoms, or relapses (Neitz, 1940).

E. wenyoni can be transmitted readily from an infected calf to a susceptible one by inoculation of infected blood. Donatien and Lestoquard (1937) believed that ticks acted as vectors, but Neitz (1940) stated that rearing of calves under tick-free conditions was no guarantee of their freedom from infection with Eperythrozoon. Foote et al. (1957) stated that infection appeared to be easily transmitted between calves by flies or possibly by direct contact.

Members of the genus Eperythrozoon possess characteristics which are not clearly protozoan in nature but which appear to be closely related to the bacteria (Peters and Wigand, 1955; Weinman, 1957).

Consequently, the genus is placed in the order Rickettsiales. The species which infects cattle has been called E. wenyoni, (Adler and Ellenbogen, 1934; Delpy and Rafyi, 1938; Neitz; 1940, Brocklesby, 1958; Hoyte, 1962) or E. wenyonii (Weinman, 1957); however, the former seems to be the more common spelling.

Very little work has been reported on the physical and chemical changes in blood as a result of bovine eperythrozoonosis. Dimopoullos et al. (1959) reported that concurrent with the appearance of parasites in the blood, serum globulins decreased to an abnormally low level. In 1964, Dimopoullos and Bedell reported the phospholipid concentration of Eperythrozoon-infected stromata to be 11.7 to 13.1 grams per 100 grams of desiccated stromata. Although this was considered to be within the normal range, the stromata remained in suspension for a prolonged period of time, indicating that the surface charge may have been altered.

Eperythrozoon infection has been reported to "interfere" with other types of infection. Tyzzer (1941) found that E. coccoides introduced into a white mouse after bartonellosis was established, promptly suppressed the latter infection so that bartonellae were no longer demonstrable in stained blood films. Foote et al. (1957) reported a similar interference between anaplasmosis and eperythrozoonosis. The reasons for such interferences have not been shown conclusively.

C. Bovine Blood Lipids

1. Plasma Lipids.

Initial studies of bovine blood lipids concerned the fatty acid composition of plasma lipids. Lipids from large quantities of plasma were separated, as far as the methods then available permitted, into the various classes of lipids. The component fatty acids in the triglyceride and sterol ester fractions were quantitated by ester-fractionation (Parry and Smith, 1936; Kelsey and Longenecker, 1941). Other workers (Garton and Duncan, 1957; Lough and Garton, 1957; Garton et al., 1961) similarly resolved the lipids in bovine plasma by column chromatography and analyzed the fatty acids from the glycerides and sterol esters by reverse-phase partition chromatography.

The more recent methods provided effective segregation of the different lipid classes (Hilditch and Williams, 1964). The component fatty acids in the triglyceride fraction from column fractionation (Garton and Duncan, 1957; Lough and Garton, 1957) resembled closely the fatty acids in cow depot fat, although the oleic acid content was lower and the stearic acid content was higher. Fatty acids of the sterol ester fraction were characterized by high contents of lineolic, linolenic, and arachidonic acids which formed nearly 70 percent of the total acids. The linoleic and linolenic acids combined as sterol esters were the natural (seed-fat) forms, in contrast to the linoleic acid of depot fat. Lough and Garton (1957) suggested that perhaps dietary

linoleic and linolenic acids which escaped hydrogenation in the rumen were preferentially esterified with cholesterol.

In recent studies of bovine plasma lipids, investigators have employed column chromatography to resolve the lipid classes and gas chromatography to detect the component fatty acids of each class (Hanahan et al., 1960; Garton et al., 1961; Duncan and Garton, 1962). These reports included fatty acid analyses of the phospholipid and free fatty acid classes. Results for the glycerides and sterol esters were roughly similar to the earlier reported compositions. The phospholipids displayed the relatively low palmitic, high stearic and prominent unsaturated C 20-22 acid concentrations which are characteristic for most animals (Hilditch and Williams, 1964).

2. Erythrocytic Lipids.

Behrendt (1957) stated that the content and partition of the plasma lipids varied considerably under the influence of absorption, diet, nutrition, and numerous metabolic processes. Erythrocytic lipids are part of the fixed framework and their primary function is of structural rather than metabolic importance; therefore, changes are not as rapid or as great as in plasma (Behrendt, 1957).

Tannhauser and Setz (1936) described a method for the quantitative determination of phospholipids in the stromata of red blood cells. These authors employed a colorimetric procedure for phosphorus to determine the total phospholipids. By using a mathematical constant,

the amount of total phospholipid was calculated. Differences in the concentration of total phospholipids were not observed when the erythrocytes from a number of disease states were compared to normal cells.

White and Mohaghan (1936) employed prolonged alcohol-ether extractions of bovine stromata for lipid analysis. Erickson et al. (1938) compared the lipids extracted from stromata prepared in a variety of ways. Using alcohol-ether extraction at room temperature, they reported a higher percentage of lipid in the stromata of the sheep and bovine erythrocytes than in the stromata of the human cells.

The analyses of Parpart and Diziemian (1940) indicated higher concentrations of lipid in bovine stromata than reported by Erickson et al. (1938) when measured as mg/ml packed cells. However, when compared on the basis of lipid content per cell, the data were in close agreement.

Paper and column chromatography have been used to separate and analyze human and bovine erythrocytic phospholipids (Turner, 1957; Turner et al., 1958). In such studies a decided difference was noted in the lipid pattern of the two species, but only limited information was offered on the chemical nature of the individual lipid components.

Dawson (1957) examined the composition of stromata and found the ratio of phosphatidylserine/phosphatidylcholine to be high in

ruminant stromata. Concurrently, Turner (1957) published a preliminary account of semi-quantitative studies on the phospholipid composition of erythrocytic stromata and noted the unusually small amount of lecithin in ruminants, in contrast with non-ruminants. These results were supported by the subsequent findings of Turner et al. (1958) and Hanahan et al. (1959).

Quantitative analyses of bovine red blood cell phospholipid composition have been carried out in several laboratories using different techniques (Dawson et al., 1960; Hanahan et al., 1960; de Gier and van Deenen, 1961; Schrader and Dimopoullos, 1963). Averages of the values obtained by four laboratories are as follows: lecithins, 12 percent; sphingomyelins, 56 percent; cephalins, 32 percent. These data confirm the original observation of Turner (1957) that the lecithin content of erythrocytes of certain ruminants is of a low order.

D. Blood Lipids in Pathologic Conditions

Behrendt (1957) gives a sobering word of caution concerning discrepancies in analytical data presented by various investigators. Both the analytic procedures and the calculations limit the accuracy of lipid determinations in blood cells and plasma. He states that the normal values, particularly for red blood cell lipids, are based on a limited number of case studies, which account for the unusually wide range of normal values. Distinct patterns of lipid distribution have been established, although the individual determinations must be

interpreted with great caution (Behrendt, 1957). Since bovine red blood cells have been studied far less than human cells, the problem of establishing normal values is made much more acute. Hanahan et al. (1960) stated that although previous observations are of considerable value, additional knowledge is needed on the chemical nature of the blood lipids in human and bovine plasma and erythrocytes.

On the foregoing premise, some lipid changes associated with disease conditions will be reviewed for possible patterns which may be helpful in this discussion. As previously mentioned, most studies on blood lipids have been conducted on human samples; consequently, this review must of necessity draw on such data. Blood studies in humans serve merely as guides because of the species differences which exist and because data from normal and disease conditions in humans may be from different individuals (Behrendt, 1957). Studies of changes in experimentally-induced infections in animals eliminate differences attributed to individual hosts by measuring changes resulting during the disease. Variations due to diet may also be controlled in animals, whereas, such control in humans is virtually impossible.

1. Lipids of Abnormal Human Erythrocytes.

The essential role of lipids in the structural organization and functioning of the red blood cell membrane lead to the assumption that alterations in the lipid moiety might cause abnormalities in shape,

permeability characteristics, metabolic activities, and life span of the erythrocytes (van Deenen and de Gier, 1964). This assumption has been the initial premise in many investigations and has led to numerous studies of blood cells in anemias. In spite of the numerous reports, definite observed abnormalities have been limited in number. It is also questionable whether the lipid defects are primarily or secondarily involved since the samples studied often contained an abnormal proportion of very young cells (van Deenen and de Gier, 1964). In several cases of hemolytic anemia, increases in lipid content could have been influenced by the increased number of reticulocytes (Erickson et al., 1937; Allison et al., 1960; Phillips and Roome, 1962).

Erickson et al. (1937) found an increase in the concentration of total lipids of the erythrocytes in erythroblastic anemia of humans. The observed changes were due to increased concentrations of neutral fats and cerebrosides. Hypochromic and hemolytic anemias resulted in decreased content of phospholipid in erythrocytes (Williams et al., 1937; Erickson et al., 1941); whereas, sickle cell and erythroblastic anemias showed higher than normal amounts of erythrocytic phospholipid (Erickson et al., 1937; Williams et al., 1940).

Williams et al. (1937, 1940) reported that in pernicious anemia the phospholipid concentration of the red blood cells was generally lower during relapse and increased with treatment. The concentration of phospholipids was above normal in the stimulated stage of recovery

which was characterized by an influx of young cells. Increases in cephalin and sphingomyelin were also noted, while the amount of lecithin decreased.

Increased concentration of total corpuscular lipid in polycythemia was demonstrated by Erickson _et al_. (1941). This was mainly due to an elevated concentration of total phospholipids as a result of a marked increase in concentrations of the cephalin and sphingomyelin fractions. Cardiac disorders caused increases in the concentrations of cephalin, lecithin and sphingomyelin (Erickson, 1940).

Red blood cells in paroxysmal nocturnal hemoglobinuria were reported to contain a significantly higher amount of both cholesterol and lipid nitrogen (Harris _et al_., 1957). The observation was confirmed by Formijne _et al_. (1957), who found the amount of cholesterol as well as the phospholipid content to be elevated. Studies on the ratios between different classes of phospholipids (Harris _et al_., 1957) indicated a decrease in lecithin and an increase in cephalin. Subsequently, however, several investigators found quite normal values for the different types of phospholipids (Formijne _et al_., 1957; Barry, 1959; Phillips and Roome, 1962).

Allison _et al_. (1960) and Kates _et al_. (1961) reported that erythrocytes from two patients with hereditary spherocytosis showed a significant decrease in cephalin content; whereas, the amount of lysocephalin was increased in comparison to normal values. When

lysocephalin was added to normal serum, sphering of normal red blood cells was observed. Consequently, the investigators concluded that the primary genetically controlled abnormality in hereditary spherocytosis probably is caused by a partial block in the enzyme system catalyzing the conversion of lysophosphatidylethanolamine to phosphatidylethanolamine. On the other hand, de Gier et al. (1961) analyzed erythrocytic phospholipids in several patients with hereditary spherocytosis and found quite normal values. They suggested that during the analyses of Allison et al. (1960), part of the cephalin (plasmalogen type) was hydrolyzed to free aldehyde and lysocephalin. Their idea was reaffirmed by Phillips and Roome (1962).

In 1961 Ways et al. reported on three cases of hereditary acanthocytosis in which normal quantities of total red cell phospholipids were found, but the lecithin content was significantly decreased with concomitant increase of the spinogomyelin fraction. Phillips (1962) recorded similar changes in four additional patients.

Red blood cell phospholipids have been studied in patients with pernicious anemia (Formijne et al., 1957; Phillips and Roome, 1962), hereditary elliptocytosis (de Gier et al., 1961) sprue, intermediate thalassemia, sicke cell anemia, and polycythemia vera (Phillips and Roome, 1962). The distributions reported in all of these studies were within the range of values accepted for normal erythrocytes (van Deenen and de Gier, 1964).

2. Lipids of Abnormal Canine Erythrocytes.

In a study of anemia produced by n-propyl disulfide in dogs, Williams et al. (1941) reported that a decrease occurred in the total phospholipid of the red blood cells. The marked change in phospholipid concentration was due to a decrease in cephalin content.

Tishkoff et al. (1953) found very little difference in the erythrocytic phospholipid content of normal dogs and dogs suffering from hemorrhagic anemia. Phospholipid changes in the erythrocytes of dogs were also described by Robscheit-Robbins and Whipple (1955). Anemia induced by blood removal and hypoproteinemia caused a rise in the level of phospholipid. Similar, but less marked changes were observed in an anemia produced by phenylhydrazine.

3. Lipids of Abnormal Bovine Erythrocytes.

Studies of blood lipids in bovine diseases have been very limited. However, some work has been reported on blood during Anaplasma infection (Dimopoullos and Bedell, 1962; Schrader and Dimopoullos, 1963). In the acute stages of anaplasmosis, Dimopoullos and Bedell (1962) found a decrease in concentration of total phospholipid in the erythrocytes. As the animals began to recover and the number of erythrocytes increased, the concentration of erythrocytic phsopholipid increased to preinfection levels. Plasma phospholipids decreased at the onset of the experimental disease but failed to return to preinfection levels.

Schrader and Dimopoullos (1963) fractionated the classes of phospholipids of erythrocytes from splenectomized calves infected with *A. marginale*. Phospholipid classes were separated by silicic acid chromatography, estimated gravimetrically, and characterized by infrared absorption spectrophotometry. They found that the total phospholipid concentration of erythrocytic stromata decreased during the anemia as a result of the significant decrease in concentrations of lecithins and cephalins. The concentration of sphingomyelins decreased only slightly. Individual phospholipids returned to preinfection levels during convalescence (Schrader and Dimopoullos, 1963).

4. Fatty Acids of Abnormal Erythrocytes.

Fatty acid constituents of abnormal red blood cells have been considered by various investigators. The fatty acid patterns of red blood cell lipids in a great number of hemolytic diseases were estimated by Munn and Crosby (1957, 1961) using ultraviolet spectrophotometry after isomerization in alkali. Lipids of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria were found to exhibit a low concentration of oleic acid, while the content of arachidonic acid was unusually high. Prankerd (1959) confirmed this observation by gas-liquid chromatography, but Leibetseder and Ahrens (1959) showed no clear-cut difference between fatty acid patterns of normal red cells and those in paroxysmal nocturnal hemoglobinuria.

Much of the data collected by Munn and Crosby (1961) on the fatty acid composition of a great number of patients with red blood cell abnormalities have varied with the individual and could not be specifically correlated with the diagnosed disease. However, a number of patients had relatively low levels of polyunsaturated fatty acids which were considered to be a consequence of immaturity of the red cell population. Earlier studies by Munn (1958) had demonstrated that the unsaturated fatty acids decreased with increasing immaturity of the cell population. He studied a patient who was being bled 2 liters per week for treatment of hemochromatosis. Similar findings were reported by de Gier et al. (1964) from studies on red cells of patients with pernicious anemia, hereditary spherocytosis, hereditary elliptocytosis, and nonspherocytic hemolytic anemia. In several patients a very low concentration of linoleic acid was found. This led van Deenen and de Gier (1964) to postulate that perhaps an increase in the manufacture of blood cells caused a local deficiency of the essential fatty acid, linoleic acid.

MATERIALS AND METHODS

A. Experimental Animals

Calves from 3 days to 3 weeks of age were obtained from local livestock auctions or from dairies in the surrounding area. They were fed nursing and starter rations until they were about 3 months old. At that time they were gradually changed to a ration consisting of 18 percent protein supplemented with a vitamin-mineral mixture. Grass hay and fresh water were available *ad lib*.

Experimental animals were separated into groups of 2-8 animals each and housed in screened barns. Rees (1933) reported that splenectomy of calves under one year of age enhanced their susceptibility to anaplasmosis. Therefore, all experimental animals were splenectomized at least 2 weeks prior to inoculation.

Anaplasmosis was experimentally produced in splenectomized calves by intravenous inoculation with 100 ml of whole citrated blood from carrier animals. Eperythrozoonosis was transmitted in a similar manner by inoculation of 100 ml of whole infected blood from an animal which had previously developed the disease following splenectomy.

B. Hematology

Blood for hematologic determinations was collected by jugular venipuncture into 12 x 100 mm glass tubes containing the dipotassium salt of ethylenediamine tetraacetic acid (EDTA) as anticoagulant.

These determinations included erythrocyte counts, percent packed cell volumes, and percent erythrocytes containing *Anaplasma* bodies. Erythrocytes were enumerated with a Coulter electronic blood cell counter and the percent packed cell volume was measured by the micro-hematocrit method. Smears of blood were prepared from each EDTA-treated sample and stained with Giemsa stain to visualize the marginal bodies or eperythrozoa. The percent of *Anaplasma*-infected erythrocytes was ascertained by counting the numbers of infected and noninfected cells.

C. Preparation of Materials

1. Preparation of Erythrocytes.

Hanahan *et al*. (1960) reported that human blood collected in heparin showed considerable alterations of the phospholipid fraction of the erythrocytes. Phosphatidylethanolamine tended to form lyso-derivatives. Therefore, blood collected in heparin was not considered to be a reliable source for "native" phospholipids from the erythrocyte (Hanahan *et al*., 1960). Based on this report, all blood for lipid studies was collected in 0.2 percent sodium citrate rather than in heparin.

Citrated blood was centrifuged in 50 ml tubes at 1860 x *g* for 15 minutes to separate the cells from plasma. The plasma and buffy coat of leucocytes were removed by aspiration and the erythrocytes were washed three times to eliminate residual plasma proteins and

leucocytes. After the final wash the packed cells were retained for lipid extraction.

2. Isolation of Marginal Bodies.

At the peak of *Anaplasma* infection, calves were exsanguinated by cannulation of the carotid artery. The blood, containing 60-80 percent infected erythrocytes was collected under partial vacuum into four-liter flasks containing sufficient heparin sodium to give a final concentration of 10 minims per 1 ml of blood.

The erythrocytes were packed and washed three times in 0.9 percent sodium chloride solution by centrifugation at 1060 x *g* for 20 minutes at 4 C. Washed, packed cells were diluted with an equal volume of 0.9 percent sodium chloride solution and 200 ml aliquots were subjected to ultrasonic vibration at 4 C for 75 seconds using a 75 watt, 20 kc Branson Sonifier. Complete lysis of all cells was ascertained by darkfield examination of the sonicate.

After ultrasonic treatment, the preparation was subjected to centrifugation at 1060 x *g* for 30 minutes at 4 C to remove large particulate matter. The supernatant fluid was aspirated and centrifuged at 105,000 x *g* for 30 minutes at 1 C. Sediment was washed three times in M/15 dibasic sodium phosphate buffer solution at pH 8.3. The washed sediment was subsequently used for lipid extraction.

D. Extraction of Lipids

All solvents were reagent grade and all except diethyl ether were redistilled prior to use. The solvents and their corresponding temperatures of distillation were as follows: petroleum ether, 40 C; chloroform, 62 C; methanol, 65 C; and benzene, 80 C. Dry methanol was prepared by distilling over calcium oxide and collecting half of the original volume.

Lipids were extracted from packed erythrocytes and marginal body preparations using modifications of the methods of Reed et al. (1960) and Ways and Hanahan (1964). Given volumes of erythrocytes or marginal bodies were slowly added to five volumes of redistilled methanol as it was stirred vigorously on a magnetic stirrer. Careful addition of the cells was mandatory to insure even dispersion and to prevent massive clumping as the proteins were being denatured. The suspension in methanol was stirred continuously for 30 minutes at 25 C and five volumes of chloroform were added as stirring was continued for an additional 10 minutes.

The extracts were filtered through 4 thicknesses of solvent-extracted filter paper in a Millipore filter assembly fitted into a vacuum filter flask. The residue was extracted twice more using the same procedure. The three filtered extracts were pooled and evaporated to complete dryness in a rotary-vacuum evaporator at 37 C. The dried residue was extracted with 3 aliquots of chloroform, equal to 10 times the original volume, filtered as before, pooled, and concentrated to 10-15 ml.

E. Thin-Layer Chromatography

Glass plates (200 x 200 mm) were thoroughly washed with detergent, rinsed with tap and distilled water, oven-dried, and cleansed with methanol prior to coating. A slurry was prepared by mixing 25 g of silica gel G with 60 ml of water for 15 seconds in a Servall Omni-Mixer. The slurry was spread in a 0.25 mm-thick film over 5 plates using a Desaga/Brinkmann adjustable applicator. After air drying for at least 30 minutes, the plates were placed in an oven at 121 C where they were kept until used.

A lane 20 mm wide was marked on one side of each thin-layer plate for separation of a standard lipid mixture containing phospholipid, cholesterol, oleic acid, triolein, and cholesterol oleate. The standard and lipid sample were spotted even with each other 10-15 mm from the bottom of each plate. The concentrated lipid sample in chloroform was applied with a glass tuberculin syringe and 27 guage needle in a series of confluent spots across the plate.

Standard Desaga tanks were filled daily with fresh solvent containing 84 parts of petroleum ether, 15 parts of diethyl ether, and 1 part of formic acid for developing thin-layer plates. A filter paper wick was placed along one side of the tank to increase the vapor concentration in the upper part of the chamber and thus shorten the developing time. The thin-layer plates were developed until the solvent front was 10-15 mm from the top.

Developed plates were flushed with nitrogen for 20-30 minutes to remove all trace of solvents, particularly formic acid. The dried plates were sprayed lightly with a 0.2 percent solution of 2',7', dichlorofluorescein in 95 percent ethanol. The lipid standards and corresponding bands in the lipid sample were delineated under short wave ultraviolet light (2537 A) and each fraction except the sterols was scraped from the glass plate into a 50 ml round bottom flask.

F. Preparation of Methyl Esters

Fatty acids in each class of lipids were converted to their methyl esters by interesterification using the method described by Stoffel *et al*. (1959) and modified by Connellan and Masters (1965). Transmethylation mixture consisting of 150 ml of dry methanol, 75 ml of benzene, and 7.5 ml of concentrated sulfuric acid was added in 15 ml aliquots to each lipid sample contained in the 50 ml round bottom flask. The flasks were heated to 100 C and refluxed for at least 2 hours. The material was then allowed to cool and each condenser was washed with about 5 ml of petroleum ether. The mixture was transferred to 20 x 150 mm glass tubes with ground glass stoppers for extraction and washing. To extract the methyl esters, 5-10 ml petroleum ether were added to each tube with an equal volume of water. The tubes were stoppered, shaken vigorously, and allowed to stand until the water and ether layers separated. The lower water and acid layer was removed with a Pasteur pipette and discarded. Water was used to wash the petroleum

ether fraction at least two more times, after which the extract was transferred to a flask containing anhydrous sodium sulfate granules to remove residual moisture. After standing 30 minutes, the methyl esters were sufficiently dry to be concentrated and injected into the gas chromatograph for analysis of fatty acid composition.

G. Gas-liquid Chromatography

During the initial phases of this study, a F & M Model 500 gas chromatograph with a thermal conductivity detector was used. Columns employed in this instrument were prepared commercially and consisted of 1/4 inch stainless steel tubing 18 feet long packed with 20 percent diethyleneglycol adipate and 2 percent phosphoric acid on acid-washed Chromsorb W, 60-80 mesh. The injection port and detector block of the instrument were maintained at 300 C and the column was held at 220 C. Helium served as the carrier gas and flowed through the column at the rate of 60 ml per minute.

Due to the lack of sensitivity of the thermal conductivity detector, the original chromatograph was replaced by a F & M Model 700 dual column gas chromatograph with flame ionization detectors. This instrument facilitated the detection of fatty acids in each class of lipids fractionated by thin-layer chromatography. Quantitative separation of fatty acids was accomplished by means of stainless steel columns measuring 1/4 inch x 10 feet packed in the laboratory with 8 percent polyethylene glycol adipate on acid-washed Chromport, 80-100 mesh.

This instrument was operated with the injection ports and detectors at 275 C and the column at 180 C. Helium flowing at the rate of 60 ml per minute served as the carrier gas through the columns. Compressed air (15 psi) and hydrogen (16 psi) were used as the gases for the detector flame.

Polyethylene glycol adipate for column packing was prepared according to the method of James (1960). A round-bottomed flask containing 65.1 g of ethylene glycol and 146 g of adipic acid was heated in silicone oil bath at 180 C for 3 hours under a slow stream of nitrogen. As soon as the ethylene glycol and adipic acid melted, 25 mg of p-toluenesulfonic acid was added as an acid catalyst. During the first 3 hours of heating, the nitrogen swept out water vapor as it was evolved. After 3 hours, the nitrogen was turned off and heating was continued for 2 more hours under vacuum to remove the residual water and escess glycol. The polymerized adipate was divided into small aliquots, allowed to solidify at room temperature, and stored in a desiccator under reduced pressure.

Packing for columns was prepared by dissolving 4 g of polyethylene glycol adipate in about 500 ml of chloroform in a round bottom flask. To this was added 46 g of Chromport with gentle mixing to insure a uniform coating. Chloroform was removed by evaporation on a rotary vacuum evaporator in a water bath at 60 C and the resulting dry material was placed in an oven at 120 C for at least 2 hours to eliminate the last traces of solvent.

Either straight lengths or previously coiled sections of stainless steel tubing were used for packing. A small plug of spun glass wool was placed in one end of the column to support the packing. Straight columns were held vertically while the powder was poured through a funnel into the tube as it was continuously vibrated. Previously coiled columns were packed in a similar manner except that a vacuum pump was attached to the distal end of the tubing to draw the packing through the coils. New columns were conditioned overnight at 180-200 C under a flow of helium. Methyl ester standards containing palmitic acid were chromatographed on the columns and their efficiencies were calculated with the formula $E = 16 \times (OH/GF)^2$ given by James (1960). The designation OH is the retention time from the injection point to the center of the peak for palmitic acid and GF is the peak width. Efficiencies of new columns ranged from 2500 to 4000.

A standard of fatty acid methyl esters was chromatographed each day prior to analyses of unknown samples. The standard contained saturated fatty acids from C 11 to C 18 and unsaturated fatty acids, C 14:1, C 16:1, C 18:1, and C 18:2. Other qualitative standards supplied by the National Institutes of Health were chromatographed to establish their relative retention times. A quantitative standard was also run to check the accuracy and sensitivity of the instrument.

Fatty acids on the chromatograms were identified by direct comparison to standards run on the same day and by comparisons of

relative retention times. The height and half-width of the base of each peak on the chromatogram was measured. These data and the attenuation of the instrument for each peak were programmed on a 7040 IBM computer to calculate the area of each peak, the total area of all peaks, and the relative percent area of each peak. The computer program is shown in Figure 1.

```
C      DR A. ROLAND DOMMERT
C      DEPT OF VETERINARY SCIENCE LSU
C      FATTY ACID COMPOSITION    9 FEB 66
       DIMENSION HGT(100),HW(100), ATN(100),AREA(100)
       DIMENSION PCA(100),LABEL(20),ID(3,100),IDA(3)
 10    DO1I=1,100
       HGT(I)=0.
       HW(I)=0.
       ATN(I)=0
       AREA(I)=0.
       PCA(I)=0.
 1     CONTINUE
       J=0
       K=0
       SUMA=0.
 13    READ2,IDA,HGTA,HWA,MLTA,LABEL
 2     FORMAT(3A2,5X,F6.2,5X,F6.2,5X,I5,2X,20A2)
       IF(HGTA)9,4,3
 4     IF(HGT(1))9,5,6
 5     PRINT12,LABEL
 12    FORMAT(1H1,40X,20A2/)
       GOTO13
 3     J=J+1
       DO15I=1,3
 15    ID(I,J)=IDA(I)
       HGT(J)=HGTA
       HW(J)=HWA
       ATN(J)=MLTA
       AREA(J)=HGT(J)*HW(J)*ATN(J)
       SUMA=AREA(J)+SUMA
       GOTO13
 6     K=K+1
       PCA(K)=(AREA(K)*100.)/SUMA
       MTL=ATN(K)
       PRINT7,K,(ID(I,K),I=1,3),HGT(K),HW(K),MTL,AREA(K),SUMA,PCA(K)
 7     FORMAT(1X,I2,2X,3A2,4X,F7.2,4X,F7.2,3X,I5,2X,
      *F14.4,2X,F14.4,2X,F7.3)
       IF(J-K)9,20,6
 9     PRINT14
 14    FORMAT(//1X21HYOU GOOFED,TRY AGAIN)
       PAUSE 1
 20    SPCA=0.
       DO21K=1,J
 21    SPCA=SPCA+PCA(K)
       PRINT22,SPCA
 22    FORMAT(75X,7H*******/74X,F8.3)
       PRINT12,LABEL
       GOTO10
       END
```

Figure 1. Program for the 7040 IBM computer to calculate the individual peak areas, total peak area, and relative percent area of each peak on fatty acid chromatograms.

RESULTS AND DISCUSSION

The original projection of research for this dissertation problem envisaged a thorough study of the lipid alterations in bovine erythrocytes associated with *Anaplasma* infection. The initial step involved the analysis of fatty acids in the total lipid extract from bovine erythrocytes throughout the course of anaplasmosis. As the study progressed, another infectious anemia, eperythrozoonosis, was encountered in a number of splenectomized, experimental calves. This disease causes a moderate to severe anemia in splenectomized calves and often exists concurrently with anaplasmosis (Foote *et al.*, 1957). Therefore, a study of the erythrocytic lipids associated with eperythrozoonosis was also included in the research project. In addition, the total fatty acid composition of bovine erythrocytes from 4 calves with dual infections of anaplasmosis and eperythrozoonosis was determined over a period of 21 days.

After surveying the fatty acids of the total lipid extracts, thin-layer chromatography was used to partition the classes so that the fatty acid composition of each class of lipids could be assayed. Erythrocytic lipids from calves with pure infections of anaplasmosis and eperythrozoonosis were studied in this manner. Finally, information on the fatty acids of the *Anaplasma* organism was obtained by

analyzing the total lipid extract and the various classes of lipids in isolated, partially-purified marginal bodies.

A. Fatty Acids of Erythrocytes from Asplenic Calves

Establishing the distribution and relative concentrations of fatty acids in erythrocytes of young, asplenic calves was necessary before pathologic changes could be determined. Data on the area percent fatty acid composition in the total lipid extract and the classes of sterol esters, triglycerides, free fatty acids, and phospholipids are presented in Table 1. The fatty acids found ranged from C 12:0 to C 24:0 with appreciable amounts of odd-numbered fatty acids. Some fatty acids were labeled with a specific carbon chain length followed by an indication that the fatty acid was unidentified. This designation was used because the fatty acids had retention times which corresponded to the carbon chain listed, but final structural analyses to determine the degree of unsaturation, number of conjugated double bonds, or branching were not made in this study.

Fatty acids found in the total lipid extract were predominantly C 16:0, C 18:0, C 18:1, C 18:2, C 22:U, and C 24:0. The component with the highest concentration was C 18:1 and the next three highest fatty acids, C 18:0, C 18:2, and C 24:0, were each found in quantities equal to about half that of oleic acid.

High concentrations of fatty acids with retention times greater than that of C 18:2 were found in the total lipid extract and the ratio

Table 1. Area percent fatty acid composition of total lipid extract and lipid classes in bovine erythrocytes from asplenic calves.

Fatty Acids	Total Lipid	Sterol Esters	Tri-glycerides	Free Fatty Acids	Phospho-lipids
C 12:0	Tr[a]	3.18	3.61	2.75	0.51
C 12:U[b]	Tr	2.00	2.82	1.99	0.78
C 13:0	Tr	2.25	4.11	2.98	1.49
C 13:U	Tr	6.25	3.38	3.51	1.16
C 14:0	0.59	5.13	5.79	4.64	0.73
C 14:1	Tr	6.80	5.21	3.91	1.12
C 15:0	0.32	6.44	3.32	3.60	1.38
C 15:U	Tr	5.40	4.18	3.78	0.70
C 16:0	7.99	7.99	12.45	14.43	15.41
C 16:1	2.59	7.17	5.25	5.88	2.03
C 17:0	1.14	5.18	4.41	3.17	2.04
C 17:U	Tr	2.14	2.59	2.97	0.68
C 18:0	12.68	4.35	12.07	9.89	22.47
C 18:1	25.99	7.14	12.47	6.69	27.79
C 18:2	15.91	5.48	6.93	9.45	7.69
C 20:U	Tr	2.63	2.80	3.25	0.99
C 20:4	2.98	3.10	7.44	4.51	3.48
C 22:0	7.15	6.30	1.49	2.20	3.48
C 22:U	9.96	4.45	0.53	0.62	2.93
C 24:0	12.71	12.10	Tr	12.76	14.95

[a]Trace amount.

[b]Unidentified fatty acid.

of saturated to unsaturated fatty acids (S/U) was calculated to be 0.90. This ratio is primarily a reflection of the high relative concentrations of unsaturated C 18 fatty acids.

Thin-layer chromatography was used to separate lipids into the classes of sterol esters, triglycerides, free fatty acids, sterols, and phospholipids. Gas-liquid chromatography was employed to analyze the fatty acid composition of all classes (Table 1) except the sterols which do not contain fatty acids.

The most impressive feature of the fatty acids in the sterol ester fraction was the striking uniformity of concentrations. Only C 24:0 constituted more than 10 percent of the total while each of the other fatty acids made up about 5 percent. The preponderance of C 18 acids noted in the total lipid extract was not apparent in this fraction.

Hanahan et al. (1960) reported on a similar study of the fatty acids in lipid classes of bovine erythrocytes. They found 61 moles percent of C 18:2 in the cholesterol ester fraction among other differences. The obvious differences in their report and these data are subject only to speculation.

Ways and Hanahan (1964) stated that differences in data from lipid analyses are partially explained by the variety of extraction and analytical procedures employed, not all of which have been definitively evaluated. Some differences in the procedures of Hanahan et al. (1960) and those used in this project are as follows:

they collected blood from mature animals, washed the erythrocytes only once, extracted with 95 percent ethanol and diethyl ether, and separated the classes of lipids on silicic acid columns. Ethanol and ether were shown by Ways and Hanahan (1964) to be much less effective for extraction of lipids from erythrocytes than methanol-chloroform used in this project. Duncan and Garton (1962) studied the fatty acid composition of lipid classes from bovine plasma and reported that the C 18 unsaturated acids constituted 80 percent of the total fatty acid fraction with C 18:2 accounting for 52.4 percent. Perhaps the high concentration of C 18:2 reported by Hanahan et al. (1960) in erythrocytes was partly due to plasma lipids adsorbed on the erythrocytes which were washed only one time.

The triglyceride fraction contained 12 percent each of C 16:0, C 18:0, and C 18:1 with about 7 percent each of C 18:2 and C 20:4. Other interesting features of this analysis were the rather even distributions of minor components and the scarcity of long-chain fatty acids beyond C 20:4.

Hanahan et al. (1960) reported a different distribution of fatty acids in bovine erythrocytic triglycerides. They found measurable amounts of C 16:0, C 18:0, C 18:1, and C 18:2 with only traces of other components. The C 16:1 represented 62 moles percent in this fraction. The probable reasons for the discrepancies in data have already been discussed.

The principal free fatty acids in bovine erythrocytes were C 16:0, C 18:0, C 18:1, C 18:2, and C 24:0 (Table 1). Each of the other component fatty acids, except C 16:1 constituted less than 5 percent of the total. The relative percentages of C 18:0, C 18:1, and C 18:2 are of interest because the free fatty acid fraction is the only class in which C 18:1 is found in lower concentration than both C 18:0 and C 18:2. Free fatty acids are present in minute quantities in red blood cells and reports on the composition of this class of lipids in animal species are lacking (van Deenen and de Gier, 1964).

Phospholipids uniformly constitute about 60 percent of the lipids from mammalian red blood cells (van Deenen and de Gier, 1964); consequently, the fatty acids in this class should closely resemble those found in the total lipid extract. Such was the case in the analyses reported in Table 1. The principal fatty acid components of the phospholipids were C 16:0, C 18:0, C 18:1, C 18:2, and C 24:0 just as in the total lipid. However, C 22:U, which constituted about 10 percent of the total lipid, made up only about 3 percent of the fatty acids found in phospholipid.

B. Erythrocytic Fatty Acids from Calves with Anaplasmosis

The primary purpose of this project was to consider the effects of anaplasmosis on the fatty acid composition of bovine erythrocytes. Progressive changes throughout the course of the disease were followed by periodic fatty acid analyses of erythrocytic lipids.

1. Total Fatty Acid Composition.

The initial phase of this study involved the analysis of the total fatty acid composition of erythrocytes during infection with A. marginale. Since the infection causes a severe anemia which is often fatal, no more than 100 ml of blood were taken from an animal at each sampling in order to minimize the added stress of bleeding. Fatty acids in this series of samples were characterized using a gas chromatograph with a thermal conductivity detector. As a result of the modest amount of lipid obtained from each sample and the moderate sensitivity of the chromatograph, lipid samples from 4 calves on corresponding days were pooled prior to analysis. The animals were inoculated on the same day and all responded to the infection in a similar manner as shown by the average hematologic values listed in Table 2.

Many interesting changes occurred in the total fatty acid composition of erythrocytes during anaplasmosis as shown in Table 2. The most obvious changes were the diminutions in the concentrations of C 16:0, C 18:0, C 18:1, and C 18:2 with a sharp increase in C 24:0 and more gradual increases in C 22:0 and C 22:U. As a guide to the changes in fatty acid content, a ratio of saturated to unsaturated acids was calculated for each analysis (Table 2). Unidentified fatty acids were not considered in the calculations. Normal erythrocytes contain approximately the same amounts of saturated and unsaturated fatty acids giving a ratio of about 1.00. As the infection progressed, the value increased

Table 2. Area percent fatty acid composition of pooled lipid extract and hematologic data from 4 calves with anaplasmosis.

Fatty Acid	Days After Inoculation								
	0	3	5	9	11	13	17	21	23
C 14:0	0.59	0.58	0.49	0.78	0.77	0.38	0.61	0.30	0.37
C 14:1	Tr[a]	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
C 15:0	0.32	0.27	0.51	0.68	1.12	0.46	0.34	0.27	0.37
C 16:0	7.99	13.77	6.77	6.25	5.82	6.71	6.11	3.60	3.80
C 16:1	2.59	2.87	1.46	1.94	2.42	1.61	1.13	0.87	1.82
C 17:0	1.14	0.80	0.52	1.24	1.54	0.89	0.47	0.49	0.74
C 17:U[b]	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
C 18:0	12.68	17.90	8.95	7.39	7.25	9.13	9.30	4.46	8.26
C 18:1	25.99	32.59	17.54	14.87	17.02	13.72	22.45	10.62	16.38
C 18:2	15.91	7.52	9.05	9.90	8.19	8.84	4.77	6.66	8.66
C 20:U	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
C 20:4	2.98	2.45	4.98	3.35	9.76	9.51	1.65	3.67	11.21
C 22:0	7.15	3.18	4.03	8.18	4.18	0.51	6.60	12.36	3.67
C 22:U	9.96	3.88	7.88	7.25	4.70	8.35	10.75	23.11	10.01
C 24:0	12.71	14.20	37.81	38.19	37.23	39.87	35.80	33.24	34.71
S/U[c]	0.90	1.12	1.79	2.09	1.55	1.72	1.97	2.51	1.36
%AB[d]	0.00	0.00	1.00	44.90	47.90	20.25	5.70	4.70	6.60
PCV[e]	31.25	28.25	27.00	16.00	10.75	10.00	19.25	23.50	25.75
RBC[f]	7.48	7.08	6.55	3.99	2.41	1.46	2.31	3.11	---

[a]Trace amount.
[b]Unidentified fatty acid.
[c]Saturated to unsaturated fatty acid ratio.
[d]Percent of erythrocytes containing _Anaplasma_ bodies.
[e]Percent packed cell volume.
[f]Erythrocyte count in millions per cu mm.

to more than 2.00 just prior to recrudescence indicating a decline in the concentration of unsaturated acids and/or an increase in saturated fatty acids. The shift is possibly due to the influence of young or immature red blood cells. Fatty acid analyses of different aged human erythrocytes indicated a slight increase in the ratio of saturated to unsaturated fatty acids (van Gastel _et al._, 1965). Conversely, Munn (1958) reported decreases in saturated acids and increased unsaturated acids as erythrocyte matured.

2. _Fatty Acid Composition of Lipid Classes._

The initial study of erythrocytic lipids during anaplasmosis was expanded to provide data on the fatty acids in individual classes of lipids separated by thin-layer chromatography. Three splenectomized calves were experimentally infected with _A. marginale_ and periodically, the red blood cell lipids from each calf were separated into sterol esters, triglycerides, free fatty acids, sterols, and phospholipids. The sterols did not contain fatty acids; consequently, they were not analyzed by gas-liquid chromatography. Each of the other fractions was chromatographed to quantitate its component fatty acids. Progress of the infections in the 3 calves is shown by the average hematologic values in Table 3. Sequential fatty acid analyses of the various lipid fractions are presented in Tables 4-7.

Table 4 presents data on the fatty acids from the sterol ester fraction. _Anaplasma_ infection in the calves caused a change in the

Table 3. Average hematologic data from 3 calves with anaplasmosis used in study of fatty acids in lipid classes.

Parameter	Days After Inoculation							
	0	3	5	7	9	10	12	13
%AB[a]	0.00	0.00	5.00	10.00	ND[d]	42.00	70.00	60.00
PCV[b]	23.67	24.33	16.83	23.00	ND	19.33	12.00	7.00
RBC[c]	6.67	ND	6.64	6.33	ND	4.85	3.98	3.96

[a]Percent of erythrocytes containing Anaplasma bodies.

[b]Percent packed cell volume.

[c]Erythrocyte count in millions per cu mm.

[d]Not determined.

Table 4. Average area percent fatty acid composition of the sterol ester fraction from erythrocytes of 3 calves with anaplasmosis.

Fatty Acid	Days After Inoculation							
	0	3	5	7	9	10	12	13
C 12:0	3.18	2.47	4.03	1.99	0.94[b]	2.08	1.03[b]	1.35[b]
C 12:U[a]	2.00	0.77	2.89	1.13	0.84[b]	1.23	1.34[b]	4.80[b]
C 13:0	2.25	1.80	3.73	0.90	0.12[b]	1.70	0.71[b]	1.44[b]
C 13:U	6.25	1.55	2.29	0.91	0.62[b]	5.69	1.50[b]	0.67[b]
C 14:0	5.13	3.99	3.76	3.40	1.87[b]	4.78	4.01[b]	5.04[b]
C 14:1	6.80	1.53	4.72	3.00	5.02[b]	4.44	2.70[b]	6.34[b]
C 15:0	6.44	3.15	6.06	2.83	1.40[b]	2.78	2.45[b]	1.51[b]
C 15:U	5.40	3.26	4.74	2.59	2.10[b]	1.62	1.61[b]	0.57[b]
C 16:0	7.99	13.70	13.86	15.98	9.72[b]	11.32	14.80[b]	16.81[b]
C 16:1	7.17	3.92	9.72	9.79	5.24[b]	9.72	8.12[b]	7.49[b]
C 17:0	5.18	3.29	3.38	1.75	1.22[b]	1.23	0.86[b]	1.35[b]
C 17:U	2.14	2.34	2.64	1.14	0.87[b]	1.87	0.67[b]	1.73[b]
C 18:0	4.35	6.65	6.79	8.31	5.39[b]	10.47	6.96[b]	9.80[b]
C 18:1	7.14	7.99	9.12	12.24	9.05[b]	11.31	11.37[b]	11.53[b]
C 18:2	5.49	6.31	10.13	6.12	23.98[b]	12.14	14.71[b]	7.80[b]
C 20:U	2.63	3.14	4.84[c]	6.27	6.81[b]	5.96	1.09[b]	1.46[b]
C 20:4	3.10	4.52	2.04[c]	5.84	2.39[b]	4.65	1.44[b]	5.04[b]
C 22:0	6.30	10.00	2.73[c]	2.66	1.94[b]	1.42	12.04[b]	2.42[b]
C 22:U	4.45[c]	5.11	1.97[c]	7.23	1.94[b]	1.65[b]	2.61[b]	1.36[b]
C 24:0	12.10[c]	14.51	6.62[c]	17.56[b]	18.55[b]	15.18[b]	9.99[b]	11.43[b]
S/U[d]	1.78	2.45	1.43	1.50	0.90	1.21	1.38	1.34

[a]Unidentified fatty acid.

[b]Single value.

[c]Average of 2 values.

[d]Saturated to unsaturated fatty acid ratio.

Table 5. Average area percent fatty acid composition of the triglyceride fraction from erythrocytes of 3 calves with anaplasmosis.

Fatty Acid	Days After Inoculation							
	0	3	5	7	9	10	12	13
C 12:0	3.61	1.97	1.46[c]	2.13	2.83[b]	3.10	0.48[b]	0.53[b]
C 12:U[a]	2.82	0.89	1.15[c]	2.67	4.52[b]	1.04	1.39[b]	2.85[b]
C 13:0	4.11	0.61	0.85[c]	2.27	0.81[b]	0.46	0.39[b]	0.49[b]
C 13:U	3.38	0.47	0.43[c]	1.62	0.69[b]	3.24	0.71[b]	0.25[b]
C 14:0	5.79	3.63	2.87[c]	3.79	3.92[b]	3.17	4.75[b]	3.43[b]
C 14:1	5.21	1.73	4.55[c]	3.15	3.98[b]	3.70	5.22[b]	1.31[b]
C 15:0	3.32	1.10	4.54[c]	1.86	2.11[b]	2.63	1.32[b]	1.10[b]
C 15:U	4.18	0.84	1.93[c]	1.51	1.09[b]	2.12	0.51[b]	0.65[b]
C 16:0	12.45	19.69	20.21[c]	19.62	21.41[b]	14.77	19.10[b]	18.35[b]
C 16:1	5.25	5.70	5.43[c]	7.33	5.14[b]	6.92	6.15[b]	6.10[b]
C 17:0	4.41	2.62	1.74[c]	3.48	1.81[b]	2.01	0.88[b]	4.24[b]
C 17:U	2.59	1.24	0.90[c]	3.67	0.72[b]	1.28	0.64[b]	0.86[b]
C 18:0	12.07	15.73	13.73[c]	12.35	18.33[b]	11.17	13.87[b]	14.27[b]
C 18:1	12.47	25.18	23.89[c]	21.55	20.05[b]	18.31	22.96[b]	30.95[b]
C 18:2	6.93	6.27	8.02[c]	5.51	7.30[b]	8.00	11.04[b]	8.02[b]
C 20:U	2.80	4.74	0.60[c]	6.49[c]	4.10[b]	12.07	2.98[b]	2.45[b]
C 20:4	7.44	3.41[c]	2.16[c]	3.04[c]	1.21[b]	1.73[c]	1.02[b]	0.55[b]
C 22:0	1.49[c]	0.97[c]	4.27[c]	1.87[b]	---	2.00[c]	0.85[b]	0.60[b]
C 22:U	0.53[b]	1.03[c]	0.75[b]	1.83[b]	---	0.98[b]	0.78[b]	0.22[b]
C 24:0	---	5.98[c]	0.52[b]	6.14[b]	---	9.62[b]	4.99[b]	2.80[b]
S/U[d]	1.27	1.24	1.14	1.32	1.05	1.27	1.01	0.98

[a]Unidentified fatty acid.

[b]Single value.

[c]Average of 2 values.

[d]Saturated to unsaturated fatty acid ratio.

Table 6. Average area percent fatty acid composition of the free fatty acid fraction from erythrcytes of 3 calves with anaplasmosis.

Fatty Acid	Days After Inoculation							
	0	3	5	7	9	10	12	13
C 12:0	2.75	0.50	1.89	1.96	1.32	1.10	0.23[b]	0.22[b]
C 12:U[a]	1.99	0.32	1.02	1.81	1.22	0.92	0.57[b]	0.51[b]
C 13:0	2.98	0.30	1.70	1.58	0.59	2.58	0.50[b]	0.14[b]
C 13:U	3.51	0.41	0.76	1.79	1.11	2.38	0.43[b]	0.41[b]
C 14:0	4.64	2.44	1.86	3.47	2.15	2.88	2.63[b]	2.56[b]
C 14:1	3.91	1.73	0.73	2.35	2.62	2.17	1.25[b]	1.66[b]
C 15:0	3.60	1.18	4.46	1.85	1.13	2.26	1.12[b]	1.60[b]
C 15:U	3.78	0.91	2.78	1.76	1.27	1.92	0.70[b]	1.25[b]
C 16:0	14.43	15.81	10.86	21.75	11.01	13.83	19.26[b]	20.94[b]
C 16:1	5.88	5.43	6.23	5.60	4.68	6.29	4.12[b]	7.08[b]
C 17:0	3.17	3.04	3.01	1.52	1.33	1.25	0.92[b]	1.49[b]
C 17:U	2.97	1.79	1.05	1.07	0.84	1.19	0.34[b]	0.93[b]
C 18:0	9.89	14.82	10.27	22.11	21.24	14.21	27.46[b]	25.73[b]
C 18:1	6.69	9.04	16.03	15.26	12.19	13.14	26.43[b]	18.66[b]
C 18:2	9.45	2.61	7.03	3.47	1.85	7.91	6.08[b]	6.62[b]
C 20:U	3.25	2.09	3.41	2.98	3.65	10.94	1.01[b]	1.85[b]
C 20:4	4.51	1.13	3.24	2.48	16.00	5.52	1.82[b]	1.25[b]
C 22:0	2.20[c]	22.35	2.58	5.64	15.73	2.04[c]	1.43[b]	1.73[b]
C 22:U	0.62[b]	6.49	4.28	0.68[c]	---	2.78[c]	0.59[b]	1.17[b]
C 24:0	12.76[b]	10.98[c]	16.15	4.03[b]	---	9.46[c]	3.12[b]	4.21[b]
S/U[d]	1.85	3.58	1.59	2.19	1.46	1.42	1.43	1.66

[a]Unidentified fatty acid.

[b]Single value.

[c]Average of 2 values.

[d]Saturated to unsaturated fatty acid ratio.

Table 7. Average area percent fatty acid composition of the phospholipid fraction from erythrocytes of 3 calves with anaplasmosis.

Fatty Acid	Days After Inoculation							
	0	3	5	7	9	10	12	13
C 12:0	0.51	0.05	0.26	0.09	0.06[c]	3.49	0.07[b]	0.01[b]
C 12:U[a]	0.78	0.08	0.44	0.16	0.02[c]	2.85	0.05[b]	0.04[b]
C 13:0	1.49	0.13	1.64	0.10	0.05[c]	0.87	0.07[b]	0.10[b]
C 13:U	1.16	0.10	0.72	0.14	0.04[c]	4.60	0.04[b]	0.18[b]
C 14:0	0.73	0.14	0.95	0.17	0.18[c]	2.84	0.19[b]	0.34[b]
C 14:1	1.12	0.14	1.52	0.17	0.07[c]	1.04	0.04[b]	0.28[b]
C 15:0	1.38	0.24	0.85	0.19	0.21[c]	3.78	0.19[b]	0.44[b]
C 15:U	0.70	0.23	0.64	0.21	0.13[c]	3.82	0.16[b]	0.44[b]
C 16:0	15.41	13.59	9.12	14.06	11.27[c]	8.87	11.40[b]	15.66[b]
C 16:1	2.03	0.90	1.09	1.40	0.94[c]	1.51	1.09[b]	0.94[b]
C 17:0	2.04	0.84	0.74	0.52	0.73[c]	0.66	0.32[b]	0.54[b]
C 17:U	0.68	0.58	0.92	5.09	0.47[c]	0.67	0.27[b]	0.31[b]
C 18:0	22.47	19.33	15.35	18.61	16.09[c]	10.77	22.75[b]	15.93[b]
C 18:1	27.79	30.79	26.86	23.32	33.25[c]	24.97	29.73[b]	31.32[b]
C 18:2	7.61	11.60	13.00	8.25	13.14[c]	11.00	10.76[b]	12.58[b]
C 20:U	0.99	0.34	1.34	1.35	0.43[c]	1.57	0.34[b]	0.28[b]
C 20:4	3.48	4.74	6.68	3.91	4.36[c]	7.11	4.47[b]	4.97[b]
C 22:0	3.48	4.10	7.17	4.33[c]	3.11[c]	3.47[c]	3.81[b]	3.60[b]
C 22:U	2.93[c]	1.06	2.58	1.05[c]	0.84[c]	1.04[c]	1.22[b]	1.05[b]
C 24:0	14.95[b]	10.98	12.17	18.56[c]	14.60[c]	9.88[c]	13.03[b]	11.00[b]
S/U[d]	1.49	1.03	0.98	1.53	0.89	0.98	1.12	0.95

[a]Unidentified fatty acid.

[b]Single value.

[c]Average of 2 values.

[d]Saturated to unsaturated fatty acid ratio.

uniform distribution of fatty acids discussed earlier in uninfected blood. For example, C 16 and C 22 increased in concentration soon after inoculation of the animals. Increased amounts of C 16:1, C 18:0, C 18:1, and especially C 18:2 may also be noted from the data. The ratio of saturated to unsaturated fatty acids was calculated each day of observation and this offers an index to some of the collective changes which occurred. The relatively high ratio which existed at day 0 increased sharply by day 3, then gradually decreased far below the normal by day 9 prior to returning to the preinoculation level. The lowest values were found to occur just prior to the peak marginal body count when the red blood cell count and the packed cell volume were decreasing rapidly.

Changes in the fatty acids of the triglyceride fraction were much greater than in the sterol ester fraction. The tabulated data (Table 5) reveal that most of the minor component fatty acids of triglycerides from uninfected erythrocytes decreased slightly through the infection, while the concentrations of the more prominent fatty acids, C 16:0, C 18:0, and particularly C 18:1, increased. C 24:0 was not detectable in the normal sample, yet during the infection it constituted up to 9.6 percent of the total fatty acids. The saturated to unsaturated ratio remained relatively stable in this lipid fraction even with the aforementioned changes which occurred. This fact helps to point out that the ratio is useful as a means of emphasizing some moderate or

small changes, yet other greater changes may have little effect on the ratio.

The profile of free fatty acids also changes during Anaplasma infection (Table 6). The changes are best summarized by noting that the fatty acids which predominate in normal erythrocytes increase in concentration during infection while the other fatty acids decrease in concentration. Specifically, the concentration of C 16:0, C 18:0, and C 18:1 increased throughout the course of the disease while C 20:U and C 20:4 showed sharp, but transient increases. The content of C 22:0 fluctuated dramatically from sample to sample. The S/U ratio changed most in this lipid fraction, quickly increasing from a pre-inoculation value of 1.85 to a high of 3.58 after which it gradually decreased during the remainder of the observation period.

Changes in the fatty acids of the phospholipid fraction were similar to those of the free fatty acid fraction in that here too, the major fatty acids increased in concentrations. The increase in major components consequently resulted in relative decreases in minor components. Such was the case with C 18:1 and C 18:2; however, C 18:0 decreased in a manner similar to the decrease noted in the total lipid extract. On the other hand, C 16:0 decreased temporarily through the infection and returned to the value found before inoculation. Since the phospholipid fraction constitutes the major class of the lipids of erythrocytes as mentioned earlier, the changes in phospholipids should

influence the total changes more than any other fraction. This pronounced influence is not evident from the S/U ratios of fatty acids from phospholipids. The S/U ratio of the phospholipid fraction dropped and remained low through the infection, whereas, the ratio in the total lipid extract increased twice during the disease.

C. Erythrocytic Fatty Acids from Calves with Eperythrozoonosis

Eperythrozoonosis has been encountered as a cause of anemia in splenectomized cattle and as a complicating factor with anaplasmosis under experimental conditions (Jensen, 1943). During the course of the present project several acute cases of eperythrozoonosis developed in splenectomized calves. Subsequently, the infection was maintained for experimental purposes by frequent passages in calves. Lipids of infected erythrocytes were analyzed to gain basic information on the molecular changes associated with the disease.

One part of the study involved fatty acid analyses of the total lipid extract of erythrocytes from calves with concomitant infections of anaplasmosis and eperythrozoonosis. Fatty acids in the various classes of lipids from erythrocytes were also determined on another series of calves with experimental eperythrozoonosis.

1. Total Fatty Acid Composition.

Four splenectomized calves were experimentally inoculated with *Anaplasma*-infected blood which also carried a latent infection of

Eperythrozoon. The calves showed evidence of concurrent infections of anaplasmosis and eperythrozoonosis 3-5 days after inoculation. The Anaplasma infection was mollified to the extent that 3 of the calves developed less than 8 percent infected erythrocytes and the fourth calf peaked at 24 percent after a lengthened incubation period (Table 8). The dual infection did not produce a precipitous drop in the packed cell volume and erythrocyte count which is associated with pure anaplasmosis, but rather a gradual decrease as shown by the hematologic values in Table 8. Eperythrozoon were only evident in blood smears for 4-6 days.

The analyses of fatty acids from these 4 calves prior to inoculation were in close agreement with the preinoculation values discussed earlier for 4 calves with anaplasmosis (Table 2). For example, the concentrations of C 16:0 and C 18:0 increased shortly after inoculation, but soon dropped to levels below the preinoculation values. The pattern for C 18:1 was much the same except that it went through two cycles. Mixed infection elicited a response in the concentration of C 24:0 which was quite different from that associated with pure anaplasmosis. The initial concentration of 14 percent dropped to 7 and was followed by a second cyclic increase to 28 percent before falling precipitously to a value of 2.0 percent. Another indication of the changes involved is the S/U ratio which remained nearly stable throughout the observation period except on day 9 when it increased over 50 percent. Subsequently, the ratio dropped below the level observed initially. The high S/U ratio on day 9 was a reflection of the high concentration of C 24:0.

Table 8. Average area percent fatty acid composition of erythrocytes and hematologic data from 4 calves with mixed infections of anaplasmosis and eperythrozoonosis.

Fatty Acid	Days After Inoculation								
	0	1	3	5	7	9	11	13	15
C 14:0	0.62	0.48	1.57	0.51	0.46	0.72	0.66	0.46	2.85
C 14:1	Tr[a]	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
C 15:0	2.32	0.32	2.36	0.47	0.61	1.13	1.30	0.79	4.04
C 16:0	9.37	10.04	10.42	7.86	10.35	7.88	9.65	10.04	9.55
C 16:1	3.93	2.69	4.22	2.65	2.55	1.97	2.44	1.74	5.03
C 17:0	1.28	0.90	1.21	1.23	1.19	1.40	1.53	1.10	4.81
C 17:U[b]	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
C 18:0	14.64	14.50	16.02	15.18	13.58	12.10	12.97	13.45	13.35
C 18:1	22.58	26.18	22.83	21.84	22.83	17.40	23.31	24.62	16.54
C 18:2	15.47	15.47	15.69	16.69	14.58	11.43	12.35	16.35	15.04
C 20:0	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
C 20:4	6.96	7.68	5.28	8.46	9.38	7.01	7.73	7.71	11.37
C 22:0	5.02	8.02	4.40	6.16	5.99	6.32	4.65	3.94	4.20
C 22:U	3.76	6.17	3.33	6.73	5.05	4.15	2.13	4.78	4.80
C 24:0	14.03	7.61	12.69	12.22	13.45	28.50	2.13	15.02	8.41
S/U[c]	0.96	0.80	1.01	0.88	0.92	1.54	0.72	0.89	0.98
%AB[d]	0.00	0.00	1.00	1.80	2.30	4.50	5.30	6.50	7.00
EPE[e]	-	-	-	±	+	+	+	±	±
PCV[f]	28.25	32.25	28.50	26.25	23.25	22.25	16.25	15.00	14.00
RBC[g]	9.84	10.20	8.97	9.28	7.34	5.40	4.33	4.55	3.11

[a]Trace amount.
[b]Unidentified fatty acid.
[c]Saturated to unsaturated fatty acid ratio.
[d]Percent of erythrocytes containing _Anaplasma_ bodies.
[e]_Eperythrozoon_ in blood.
[f]Percent packed cell volume.
[g]Erythrocyte count in millions per cu mm.

2. Fatty Acid Composition of Lipid Classes.

Two splenectomized calves were inoculated with *Eperythrozoon*-infected blood to experimentally produce an acute infection. A heavy parasitemia occurred in about 7 days which was followed by a moderately severe anemia as indicated by the hematologic values listed in Table 9. The organisms were observed in blood smears for a period of 10 days. Lipids from erythrocytes of the 2 animals were extracted throughout the initial parasitemia and fractionated into classes by thin-layer chromatography for analysis of the fatty acids.

The sterol ester fraction of lipids contained four major components, namely C 16:0, C 18:0, C 18:1, and C 18:2 (Table 10). Other components each constituted less than 5 percent of the total, except C 16:1 which contributed 8.73 percent of analysis. During eperythrozoonosis, the concentration of C 18:2 changed more than any other fatty acid by increasing from 13.51 percent to a high value of 47.07 percent. Smaller variations occurred in the concentrations of C 12:0 through C 17:U. The content of C 18:0 and C 18:1 decreased during most of the observation period; however, both fatty acids were present in higher concentrations on day 17. The S/U ratio fluctuated through two cycles during the infection.

Triglycerides of erythrocytes from the two calves with eperythrozoonosis contained C 16:0 as the predominant fatty acid throughout most of the period of observation (Table 11). This fraction was also

Table 9. Average hematologic data from 2 calves with eperythrozoonosis used in study of fatty acids in lipid classes.

Parameter	Days After Inoculation									
	0	3	5	7	10	12	15	17	19	22
PCV[a]	29.50	30.50	28.50	27.50	27.50	27.50	ND[d]	ND	20.50	24.00
RBC[b]	9.40	8.97	8.49	7.88	7.34	7.51	ND	ND	ND	6.11
EPE[c]	-	-	-	±	+	+	+	±	-	-

[a]Percent packed cell volume.

[b]Erythrocyte count in millions per cu mm.

[c]*Eperythrozoon* in blood.

[d]Not determined.

Table 10. Average area percent fatty acid composition of the sterol ester fraction from erythrocytes of 2 calves with eperythrozoonosis.

Fatty Acid	Days After Inoculation									
	0	3	5	7	10	12	15	17	19	22
C 12:0	1.09	1.61	3.92	2.17	2.67	3.06	3.14	1.33	2.80	1.96
C 12:U[a]	1.76	3.03	3.14	1.90	2.26	2.65	1.46	3.28	2.15	2.68
C 13:0	1.26	2.30	2.04	2.11	2.27	2.40	1.33	0.64	1.67	1.57
C 13:U	1.89	2.34	2.25	1.56	1.26	1.07	1.03	0.47	1.33	1.29
C 14:0	2.86	4.19	3.92	5.59	3.74	2.74	3.22	2.35	3.99	3.28
C 14:1	3.02	3.24	3.48	4.58	3.13	2.18	1.91	1.71	3.39	3.11
C 15:0	4.16	2.52	4.36	4.94	3.14	2.14	1.61	2.07	3.06	3.71
C 15:U	2.27	3.16	4.23	3.39	2.44	2.78	1.73	1.30	2.71	2.88
C 16:0	11.06	7.93	13.41	17.13	18.99	11.70	11.40	13.32	13.46	12.79
C 16:1	8.73	6.46	12.78	13.06	10.32	7.53	5.98	7.06	13.85	14.78
C 17:0	4.07	3.90	4.67	2.73	2.95	1.54	0.73	1.77	1.89	2.35
C 17:U	1.97	3.21	3.32	2.33	2.85	3.78	1.52	1.54	1.53	1.99
C 18:0	11.72	6.52	5.84	9.46	9.37	6.84	3.47	19.35	6.33	7.60
C 18:1	15.48	7.56	7.40	11.92	11.12	9.24	9.93	14.49	9.48	11.56
C 18:2	13.51	7.03	9.44	6.07	11.44	20.24	47.07	17.68	11.15	12.37
C 20:U	3.68	2.20	2.67	2.49	1.98	6.25	2.72	1.92	2.32	3.09
C 20:4	4.81	12.79	5.44	3.84	4.71	1.68	0.99[b]	3.53	4.26	6.20
C 22:0	2.40	8.37	2.89	1.18	1.36	2.60	0.29[b]	1.07	5.39	2.71
C 22:U	3.20	3.94	2.94	1.10	1.64[b]	6.19	0.20[b]	4.96[b]	2.18	2.43
C 24:0	2.16[b]	7.66	1.88	2,44	6.34[b]	3.40	1.99[b]	5.29[b]	14.13[b]	3.31[b]
S/U[c]	0.90	1.21	1.11	1.21	1.25	0.89	0.42	1.06	1.25	0.82

[a]Unidentified fatty acid.

[b]Single value.

[c]Saturated to unsaturated fatty acid ratio.

Table 11. Average area percent fatty acid composition of triglyceride fraction from erythrocytes of 2 calves with eperythrozoonosis.

Fatty Acid	Days After Inoculation									
	0	3	5	7	10	12	15	17	19	22
C 12:0	2.36	3.32	1.56	2.24	1.71	0.27	1.82	0.97	1.86	1.43
C 12:U[a]	2.05	4.32	1.77	1.87	1.05	0.42	1.08	0.56	0.45	1.82
C 13:0	1.19	2.21	2.02	1.51	1.37	0.40	1.08	0.60	1.38	0.85
C 13:U	1.19	1.73	3.18	0.98	1.39	0.19	0.30	0.35	0.36	1.51
C 14:0	5.46	4.21	3.08	4.55	2.73	0.92	2.37	1.86	5.35	3.68
C 14:1	3.78	3.53	2.43	2.77	2.37	0.35	0.99	0.85	1.88	1.96
C 15:0	3.42	2.05	2.23	2.70	2.63	0.70	1.40	1.27	3.73	2.48
C 15:U	2.10	1.79	1.33	1.51	1.36	0.38	0.86	0.78	1.22	1.25
C 16:0	19.91	18.15	15.65	17.86	20.43	13.10	22.52	17.07	19.86	17.82
C 16:1	11.15	7.85	6.42	9.43	5.76	1.86	9.98	3.46	7.33	10.32
C 17:0	4.32	5.55	3.49	4.76	3.99	1.20	6.20	2.05	6.11	3.29
C 17:U	2.57	2.14	1.76	1.94	1.16	0.74	1.53	0.62	1.74	1.05
C 18:0	9.13	10.44	9.84	10.63	13.91	22.44	22.65	21.13	11.58	10.56
C 18:1	16.01	15.08	17.78	18.25	13.78	28.89	17.08	27.79	20.02	19.74
C 18:2	6.56	5.33	10.14	6.07	6.58	16.37	7.71	12.52	5.92	6.39
C 20:U	0.72	4.83	1.70	2.72	2.59	0.89	0.56	1.22	2.01	4.51
C 20:4	3.45	1.83	2.46	1.74	8.86	8.82	0.93	1.37	1.17	1.58
C 22:0	1.00	2.46	2.08	1.61	2.48	0.80[b]	0.73	0.64	4.74	0.87
C 22:U	0.73	2.13[b]	7.02	1.43	1.00	0.79[b]	0.34[b]	2.18	2.30	4.06
C 24:0	2.91	4.28[b]	4.03	5.30	4.37	2.55[b]	1.04[b]	2.67	2.00[b]	4.93
S/U[c]	1.21	1.57	1.12	1.34	1.44	0.76	1.63	1.05	1.23	1.15

[a]Unidentified fatty acid.

[b]Single value.

[c]Saturated to unsaturated fatty acid ratio.

composed of relatively high concentrations of C 16:1, C 18:0, C 18:1, and C 18:2. The levels of C 18:0 and C 18:1 greatly increased as a result of the disease. Several of the minor components, both short- and long-chain fatty acids such as C 14:0, C 22:0, and C 22:U varied only a few percent from the start. However, when compared to the preinoculation values, these increases were as high as 2 to 3 fold. The S/U ratio in this fraction remained relatively stable.

Three fatty acids, C 16:0, C 18:0, and C 18:1, constituted almost half of the free fatty acid class of erythrocytic lipids from Eperythrozoon-infected calves (Table 12). Concentrations of C 16:0 diminished to about 50 percent of the preinoculation level when the organisms were observed in the blood, however after the parasitemia disappeared, the concentration of this fatty acid returned approximately to its initial value. In the period of observation, the level of C 18:0 increased progressively from about 10 percent to a high value of 43.95 percent. Some of the long-chain fatty acids which were minor components also varied in concentration as a result of the disease. For example, C 22:0 increased 13 fold by 12 days after inoculation. The primary reason for the high S/U ratio at that stage of the disease was due to the high concentration of C 22:0 and the drastic decrease in the concentration of C 18:1. Shortly afterward, the level of C 22:0 decreased and the content of C 18:0 reached maximum, thereby maintaining a high S/U ratio.

Table 12. Average area percent fatty acid composition of the free fatty acid fraction from erythrocytes of 2 calves with eperythrozoonosis.

Fatty Acid	Days After Inoculation									
	0	3	5	7	10	12	15	17	19	22
C 12:0	3.32	1.92	2.29	1.94	1.39	0.60	0.93	1.08	1.63	0.96
C 12:U[a]	0.94	1.52	2.10	1.62	1.22	2.63	0.44	1.31	1.25	1.71
C 13:0	0.66	0.66	1.88	1.27	1.48	1.36	0.52	0.69	1.79	0.63
C 13:U	0.54	1.00	1.21	0.82	1.31	0.74	0.58	0.94	1.44	0.51
C 14:0	4.59	3.41	4.24	2.76	2.65	3.30	1.95	2.91	3.51	4.25
C 14:1	2.92	1.90	2.43	1.68	1.89	2.00	0.74	1.30	3.34	1.59
C 15:0	2.85	3.03	2.48	1.81	1.94	2.57	1.38	2.07	2.89	3.87
C 15:U	1.20	1.04	1.56	0.62	0.75	1.78	0.50	1.45	1.73	1.08
C 16:0	24.27	12.04	23.11	13.41	17.52	11.96	13.28	15.92	19.18	23.38
C 16:1	7.67	3.10	7.76	5.40	4.71	2.16	2.71	7.81	7.00	9.76
C 17:0	2.37	2.30	3.26	1.78	2.88	1.33	1.60	1.35	2.96	2.36
C 17:U	1.65	1.68	2.25	0.89	1.35	1.53	0.56	1.91	1.97	1.32
C 18:0	9.98	18.87	12.49	13.63	17.75	16.53	43.95	10.06	15.26	16.42
C 18:1	19.70	23.64	13.67	18.60	19.89	7.86	15.85	15.97	14.73	18.70
C 18:2	4.60	7.58	5.35	8.02	8.02	10.20	6.49	25.13	5.35	5.01
C 20:U	2.78	3.92	7.21	1.42	1.72	4.79	0.67	1.22	4.08	2.15
C 20:4	1.45	3.09	1.07	2.47	2.56	3.99	2.41	1.87	1.25	1.32
C 22:0	1.29	1.93	1.40	3.40	3.54	17.25	1.93	2.71	3.17	1.80
C 22:U	2.12	0.93	1.41	1.77	0.79	0.65	1.41	0.68	9.26[b]	0.62[b]
C 24:0	4.86	6.42	2.81	16.68	6.67	6.88	4.53[b]	3.59	5.42[b]	6.24[b]
S/U[c]	1.49	1.29	1.78	1.57	1.51	2.36	2.48	0.78	1.76	1.65

[a]Unidentified fatty acid.

[b]Single value.

[c]Saturated to unsaturated fatty acid ratio.

Erythrocytic phospholipids during eperythrozoonosis contained only minute amounts of fatty acids with chain lengths shorter than 16 carbons (Table 13). The major fatty acid components of the phospholipid fraction were C 16:0, C 18:0, C 18:1, C 18:2, and C 24:0. The amount of C 16:0 and C 18:1 remained fairly constant throughout the period of study, whereas C 18:0 fluctuated considerably. The other major fatty acid, C 24:0, decreased during the infection but approached the preinfection level at the end of the observation period. The S/U ratio dropped from 1.16 at the time of inoculation and remained below this value.

D. Analyses of Fatty Acids from Isolated *Anaplasma* Bodies

Most of the investigations of anaplasmosis have been conducted on populations of erythrocytes during experimentally-induced infections. Such studies are useful in determining the effects or results of *Anaplasma* infection, but they offer little information on the organism *per se* since it constitutes such a small part of the mass of infected erythrocytes or erythrocytic stromata. Analyses of the isolated, purified organisms are the only way to unequivocally determine their composition and properties. There is no method available for isolating *Anaplasma* which are completely free of contaminating erythrocytic stromata; however, highly purified preparations may be obtained using ultrasonic treatment of infected erythrocytes and differential centrifugation as previously described. Such preparations of marginal bodies appear

Table 13. Average area percent fatty acid composition of the phospholipid fraction from erythrocytes of 2 calves with eperythrozoonosis.

Fatty Acid	Days After Inoculation									
	0	3	5	7	10	12	15	17	19	22
C 12:0	0.05	0.18	0.06	0.06	0.06	0.06	0.01	0.05	0.09	0.06
C 12:U[a]	0.04	0.10	0.09	0.09	0.09	0.04	0.05	0.10	0.03	0.11
C 13:0	0.64	1.13	0.08	0.10	0.08	0.10	0.06	0.14	0.06	0.08
C 13:U	0.05	0.17	0.08	0.13	0.09	0.04	0.08	0.10	0.08	0.09
C 14:0	0.27	0.32	0.22	0.26	0.34	0.25	0.18	0.21	0.15	0.18
C 14:1	0.07	0.31	0.12	0.17	0.14	0.10	0.08	0.12	0.08	0.09
C 15:0	0.33	0.34	0.30	0.30	0.32	0.39	0.30	0.17	0.18	0.22
C 15:U	0.28	0.32	0.30	0.26	0.35	0.25	0.19	0.20	0.20	0.49
C 16:0	10.57	10.69	10.96	8.41	11.63	11.97	14.21	11.52	11.16	10.44
C 16:1	1.50	1.54	1.37	1.08	1.31	1.19	0.85	1.18	1.30	1.20
C 17:0	0.90	0.94	1.01	0.82	1.02	0.99	1.01	0.68	0.71	0.69
C 17:U	0.56	0.59	0.71	0.50	0.60	0.41	0.34	0.47	0.50	0.55
C 18:0	18.05	14.01	14.16	16.63	20.59	18.80	20.32	11.22	13.38	16.50
C 18:1	25.90	27.32	26.83	26.87	15.43	24.00	19.06	26.33	27.89	24.26
C 18:2	10.32	18.44	16.44	16.81	17.67	17.55	19.35	14.03	17.08	18.36
C 20:U	0.42	1.22	0.43	0.56	1.04	1.24	1.39	0.32	0.53	0.54
C 20:4	5.38	6.61	5.96	3.90	8.08	7.26	9.66	6.29	7.24	6.17
C 22:0	3.54	3.47	3.77	3.75	3.87	2.74	2.50	4.64	3.36	3.50
C 22:U	0.99	0.82	1.26	1.07	1.47	0.18	0.88	1.57	1.02	1.06
C 24:0	15.85	9.12	11.65	11.66	11.27	8.09	7.23	16.42	11.37	12.12
C 24:1	4.89	2.36	4.20	3.57	4.53	3.56	2.25	4.23	3.54	3.24
S/U[b]	1.16	0.74	0.83	0.86	1.15	0.87	0.94	0.94	0.75	0.87

[a]Unidentified fatty acid.

[b]Saturated to unsaturated ratio.

practically free of contaminating stromata when examined by dark-field or electron microscopy.

Partially-purified Anaplasma bodies were prepared in this manner and the lipids were extracted for fractionation by thin-layer chromatography and subsequent fatty acid analysis. Part of the unfractionated lipid extract and each lipid class separated by thin-layer chromatography was esterified for fatty acid analysis by gas-liquid chromatography. Results of these analyses are presented in Table 14. The major components found, namely C 16:0, C 18:0, C 18:1, and C 18:2, accounted for over 86.00 percent of the fatty acids of the total lipid extract and the phospholipid fraction. By far the greatest portion is due to C 18:1 which constitutes over 40 percent of the 2 fractions. Fatty acids other than the 4 mentioned, ranged from C 12:0 to C 24:0 and were found in much smaller quantities.

The triglyceride fraction was comprised of fatty acids C 12:0 to C 18:2 with the C 16 and C 18 series predominating. This fraction also contained over 5 percent of C 14:0 which was the highest concentration of that fatty acid found in any of the fractions.

The free fatty acid fraction contained prominent amounts of C 16:0, C 16:1, C 18:1, and C 20:4 with smaller amounts of other fatty acids from C 12:0 to C 20:U. High concentrations of C 16:1 and C 20:4 were the most interesting features of this class of lipids from A. marginale.

Table 14. Area percent fatty acid composition of total lipid and lipid classes in <u>Anaplasma marginale</u>.

Fatty Acids	Total Lipid	Sterol Esters	Free Fatty Acids	Tri-glycerides	Phospho-lipids
C 12:0	0.08	1.54	2.09	0.97	0.03
C 12:U[a]	0.01	2.51	1.26	0.97	0.09
C 13:0	0.01	2.05	0.63	1.98	0.04
C 13:U	0.02	2.62	2.04	0.84	0.05
C 14:0	0.61	4.22	2.30	5.13	0.55
C 14:1	0.20	5.50	1.25	2.89	0.37
C 15:0	0.38	4.84	4.24	2.26	0.31
C 15:U	0.43	5.22	5.65	3.04	0.20
C 16:0	19.65	8.70	20.42	29.82	16.81
C 16:1	3.39	9.83	11.94	11.43	3.33
C 17:0	0.61	1.54	1.05	4.10	0.62
C 17:U	0.85	7.22	5.86	1.90	0.85
C 18:0	11.73	4.15	5.07	6.12	10.59
C 18:1	44.78	11.28	15.08	18.23	48.09
C 18:2	10.16	1.02	2.93	10.33	11.22
C 20:U	0.19	3.89	1.89	Tr[b]	0.28
C 20:4	3.57	9.67	16.34	Tr	4.57
C 22:0	0.67	4.09	Tr	Tr	0.59
C 22:U	0.31	8.48	Tr	Tr	0.19
C 24:0	2.36	1.64	Tr	Tr	1.22

[a]Unidentified fatty acid.

[b]Trace amount.

Fatty acids in the sterol ester fraction were more evenly distributed than in any other of the lipid fractions. Only one fatty acid contributed more than 10 percent of the total. Consequently, many of the other fatty acids appeared in relatively high concentrations.

E. General Discussion

The primary purpose of this investigation was to gather basic data on the fatty acid content of erythrocytes from splenectomized calves prior to and during experimental infections of *A. marginale* and *E. wenyoni*. The moderate to severe anemias associated with these diseases are accompanied by surface alterations in the circulating erythrocytes (Neitz, 1940; Dimopoullos and Bedell, 1961, 1962, 1965; Dimopoullos, 1964). However, only limited information is available on the mechanism of the anemia at the molecular level. Lipids are essential constituents of the red cell membrane and knowledge of the exact chemical structure of the members of various lipid classes as well as their proportions is a strict requirement for definite formulation of the molecular structure (van Deenen and de Gier, 1964). This investigation was designed to obtain data which would be helpful in understanding the molecular composition of bovine erythrocytes and the alterations which occurred during the anemias.

The fatty acid patterns of human red blood cell lipids have been investigated in a number of hemolytic diseases by Munn and Crosby (1957; 1961). Many deviating values were recorded, but these could

not be correlated with a specific disease. However, in a number of cases they found a relatively low level of linoleic acid which was considered to be a consequence of immaturity of the erythrocyte population. Similar findings were reported by de Gier et al. (1964) for erythrocytes associated with pernicious anemia, hereditary spherocytosis, hereditary elliptocytosis, and nonspherocytic hemolytic anemia.

Table 15 relates the fatty acid composition of the total lipid extract of normal and *Anaplasma*-infected erythrocytes to partially-purified marginal bodies. The concentrations of C 20:4 and C 24:0 increased during infection, whereas nearly all of the other fatty acids decreased. The changes do not appear to be merely the result of the contribution of the structural lipids of marginal bodies within the erythrocytes since the composition of the marginal body differs greatly from that of the infected cell. Decreased concentrations of the C 18 series could be interpreted, in the light of the preceding discussion, to mean that at least part of the changes may have been due to the presence of immature red blood cells. On the other hand, the decrease in concentration of C 16:0 and the decrease in concentration of lecithin reported by Schrader and Dimopoullos (1963) could imply an increased permeability of the cell membrane as suggested by van Deenen and de Gier (1964).

The high concentration of C 24:0 in erythrocytes during anaplasmosis is interesting because van Deenen and de Gier (1964) report

Table 15. Area percent fatty acid composition of total lipid extract from normal bovine erythrocytes, *Anaplasma*-infected bovine erythrocytes, and partially-purified marginal bodies.

Fatty Acid	Normal Erythrocytes	Infected Erythrocytes	Marginal Bodies
C 14:0	0.61	1.77	0.61
C 14:1	Tr[a]	Tr	0.20
C 15:0	1.32	1.12	0.38
C 15:U	Tr	Tr	0.43
C 16:0	8.68	5.82	19.65
C 16:1	3.26	2.42	3.39
C 17:0	1.21	1.54	0.61
C 17:U	Tr	Tr	0.85
C 18:0	13.66	7.25	11.73
C 18:1	24.29	17.02	44.78
C 18:2	15.69	8.19	10.16
C 20:U	Tr	Tr	0.19
C 20:4	4.97	9.77	3.57
C 22:0	6.08	4.19	0.67
C 22:U	6.86	4.70	0.31
C 24:0	13.37	37.23	2.36

[a]Trace amount.

[b]Unidentified fatty acid.

that C 24:0 is a very characteristic fatty acid of the glycolipids. Earlier studies of erythrocytic stromata from Anaplasma-infected calves revealed increased concentrations of sialic acids (Dommert, 1963), components of a type of glycolipid. The companion data suggest that the glycolipids of the infected erythrocyte were altered during the disease. Perhaps future investigations in this area will aid in understanding the mechanisms of hemolytic anemias, particularly those of infectious etiology.

Information on progressive fatty acid changes in various lipid classes throughout the course of infectious anemias is virtually absent in the literature. The mechanisms of such changes are also lacking; consequently, formulation of theories on these changes must await further investigations and evaluation of data. In the case of the two diseases studied in this project, more detailed studies will be necessary to elucidate the factors influencing the alterations. Perhaps the increase in body temperature of the host during infection causes alterations in enzyme rates or perhaps the invading organisms possess enzymes which are responsible for modifying the host cells. The presence of enzymes such as catalase (Wallace and Dimopoullos, 1965a), cholinesterase (Wallace, 1966), and lactic dehydrogenase (Darré, 1966) have been associated with A. marginale. These factors acting alone or in combination may play a role in altering the erythrocytic membranes so that

their structural integrity is affected. Another possibility is that the alterations are sufficient to make the erythrocytic membranes antigenic, thus initiating an autoimmune response as suggested by Ristic (1960).

SUMMARY AND CONCLUSION

Lipids were extracted from erythrocytes of 4 splenectomized calves throughout the course of experimental anaplasmosis. The lipids from corresponding days were pooled, converted to methyl esters, and analyzed by gas-liquid chromatography. The predominant fatty acids were found to be C 16:0, C 18:0, C 18:1, C 18:2, C 22:U, and C 24:0. In the preinoculation pool, C 18:1 comprised 25.99 percent of the total composition, whereas during the height of parasitism C 24:0 became the principal fatty acid, contributing almost 40 percent of the total fatty acids. *Anaplasma* infection also produced drastic decreases in concentrations of C 18:0, C 18:2, and C 22:0 in the total lipid fractions. The S/U ratio of fatty acids increased markedly as a result of the infection, reflecting the increase in concentrations of saturated fatty acids and/or the decrease in concentrations of unsaturated fatty acids.

Erythrocytic lipids from another series of 3 calves infected with *A. marginale* were fractionated into sterol esters, triglycerides, free fatty acids, and phospholipids by thin-layer chromatography. The fatty acid composition of each fraction was assayed and data from corresponding days averaged. The greatest changes were reflected by the increases in the content of C 18 saturated and unsaturated fatty acids and C 22:0. Only the triglyceride fraction exhibited increased

concentrations of C 24:0 somewhat approximating those values observed in the pooled samples of the total lipid extracts.

Dual infections of anaplasmosis and eperythrozoonosis were investigated in 4 calves. Fatty acid analyses were conducted on erythrocytic lipids collected during the first acute phase of the concomitant infections. The alterations in fatty acid composition were minimal, with most of the changes occurring in C 20:4, C 22:0, C 22:U, and C 24:0.

Fatty acids in the classes of erythrocytic lipids were determined from 2 calves experimentally infected with _E. wenyoni_. Most of the fatty acids of carbon chain lengths shorter than 18 showed minor variations or slight decreases in concentrations, whereas the longer chain fatty acids tended to show the greater increases in concentration.

Anaplasma marginal bodies were isolated from infected erythrocytes, purified by differential centrifugation, and the lipids extracted and analyzed. Thin-layer chromatography of the lipid extract separated the sterol esters, triglycerides, free fatty acids, sterols, and phospholipids. Fatty acids were assayed in the total lipid extract and in each class, with exception of the sterol fraction. The 16 and 18 carbon chain fatty acids accounted for approximately 85 percent of the total fatty acids of the unfractionated lipid, with C 18:1 accounting for 45 percent. Fatty acids in the sterol ester fraction were rather uniformly distributed, but the other three fractions contained higher concentrations

of the C 16 and C 18 series which were similar to the values found in the total lipid. Phospholipids resembled the total lipid in fatty acid composition more closely than any other class. The concentration of C 18:1 present in the phospholipid accounted for 48 percent of the total lipid analyzed.

These data substantiate earlier evidence that the lipids of bovine erythrocytes are altered during anaplasmosis. Fatty acid composition of the total lipid extract and various classes were found to fluctuate in nearly all cases, and significant trends were evident in many instances. This project was not designed to determine if the lipid alterations were the result of the host response to anemia or if the alterations were due specifically to the activity of the parasitizing organisms. Data gathered here should offer valuable background information for such studies in the future.

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VITA

Arthur Roland Dommert was born in Crowley, Louisiana on April 3, 1937. He was graduated from Crowley High School in 1955 and entered the A. & M. College of Texas that Fall. He received his Bachelor of Science degree in Animal Science in January, 1960 and his Doctor of Veterinary Medicine degree in May, 1961.

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EXAMINATION AND THESIS REPORT

Candidate: Arthur Roland Dommert

Major Field: Microbiology

Title of Thesis: Fatty Acid Analyses of Bovine Erythrocytes During Anaplasmosis and Eperythrozoonosis.

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

May 7, 1966