1973

Atpase Activity Associated With Bovine Erythrocyte Membranes in Anaplasmosis.

Hollis Utah Cox
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_disstheses/2453

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeib Road
Ann Arbor, Michigan 48106
Atpase activity associated with bovine erythrocyte membranes in anaplasmosis.

The Louisiana State University and Agricultural and Mechanical College, Ph.D., 1973
Microbiology

University Microfilms, A XEROX Company, Ann Arbor, Michigan

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED.
ATPASE ACTIVITY ASSOCIATED WITH BOVINE ERYTHROCYTE MEMBRANES IN ANAPLASMOSIS

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

by

Hollis Utah Cox
B.S., Oklahoma State University, 1965
D.V.M., Oklahoma State University, 1967
August, 1973
ACKNOWLEDGMENT

I wish to express sincere appreciation to Dr. L. T. Hart for his encouragement, suggestions, and evaluations during the experimental work and in preparation of this dissertation.

Special thanks is given to Dr. G. T. Dimopoulos for his cooperation and guidance.

Sincere appreciation is extended to my wife and my parents, Mr. and Mrs. H. R. Cox, for their support, encouragement, and understanding.

Acknowledgment is made to the Departments of Veterinary Science and Microbiology and to the Agricultural Experiment Station of Louisiana State University. Funds which supported this work were derived from the National Institutes of Health and the National Defense Education Act.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>SELECTED LITERATURE</td>
<td>4</td>
</tr>
<tr>
<td>A. HISTORICAL ASPECTS</td>
<td>4</td>
</tr>
<tr>
<td>B. TAXONOMICAL ASPECTS</td>
<td>5</td>
</tr>
<tr>
<td>C. CLINICAL ASPECTS</td>
<td>6</td>
</tr>
<tr>
<td>D. BIOLOGICAL ASPECTS</td>
<td>7</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>22</td>
</tr>
<tr>
<td>A. EXPERIMENTAL ANIMALS</td>
<td>22</td>
</tr>
<tr>
<td>B. HEMATOLOGY</td>
<td>22</td>
</tr>
<tr>
<td>C. EXPERIMENTAL INDUCTION OF ANEMIA</td>
<td>23</td>
</tr>
<tr>
<td>D. PREPARATION OF MATERIALS</td>
<td>24</td>
</tr>
<tr>
<td>1. ERYTHROCYTE MEMBRANES</td>
<td>24</td>
</tr>
<tr>
<td>2. MARGINAL BODIES</td>
<td>25</td>
</tr>
<tr>
<td>3. ANTI-ERYTHROCYTIC MEMBRANE SERUM</td>
<td>26</td>
</tr>
<tr>
<td>E. MEASUREMENT OF ATPASE ACTIVITY</td>
<td>26</td>
</tr>
<tr>
<td>1. GENERAL</td>
<td>26</td>
</tr>
<tr>
<td>2. STANDARD ASSAY</td>
<td>27</td>
</tr>
</tbody>
</table>

iii
<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Substrate specificity of ATPase associated with erythrocyte membranes from normal calves</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>Substrate specificity of ATPase associated with erythrocyte membranes from calves infected with <em>A. marginale</em></td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>Effects of magnesium, sodium, and potassium on ATPase activity associated with erythrocyte membranes prepared from normal calves and calves infected with <em>A. marginale</em></td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>Effect of varying sodium and potassium ion concentrations on the magnesium-dependent ATPase activity associated with erythrocyte membranes prepared from normal and <em>A. marginale</em>-infected calves</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>Effects of magnesium, sodium, and potassium ions on ATPase activity associated with erythrocyte membranes prepared from normal and <em>A. marginale</em>-infected calves in the presence and absence of 0.5 mM ouabain</td>
<td>46</td>
</tr>
<tr>
<td>6</td>
<td>Effect of ouabain on magnesium-dependent and total ATPase activity associated with erythrocyte membranes prepared from normal and <em>A. marginale</em>-infected calves</td>
<td>47</td>
</tr>
<tr>
<td>7</td>
<td>Effect of calcium on magnesium-dependent and total ATPase activity associated with erythrocyte membranes prepared from normal and <em>A. marginale</em>-infected calves</td>
<td>49</td>
</tr>
<tr>
<td>8</td>
<td>Activation of ATPase activity associated with erythrocyte membranes prepared from normal and <em>A. marginale</em>-infected calves by calcium in the presence and absence of 0.5 mM ouabain</td>
<td>51</td>
</tr>
<tr>
<td>9</td>
<td>Effects of various concentrations of magnesium on ATPase activity associated with erythrocyte membranes prepared from normal and <em>A. marginale</em>-infected calves in the presence and absence of 0.5 mM ouabain</td>
<td>52</td>
</tr>
<tr>
<td>Page</td>
<td>Table Title</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>Effect of ATPase inhibitors on ATPase activity associated with erythrocyte membranes prepared from calves infected with <em>A. marginale</em></td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>Effect of experimental anemia induced by phenylhydrazine injections and phlebotomy on total ATPase activity associated with erythrocyte membranes prepared from normal calves</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>Changes in total ATPase activity resulting from the incubation of erythrocyte membranes prepared from normal and <em>A. marginale</em>-infected calves with various rabbit antiseraums</td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>ATPase activity of preparations of partially-purified <em>A. marginale</em> and erythrocyte components obtained by sucrose density gradient centrifugation</td>
<td></td>
</tr>
<tr>
<td>FIGURE</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Release of H+ ions during ATP hydrolysis by ATPase associated with erythrocyte membranes prepared from calves infected with <em>A. marginale</em> at different concentrations of membrane protein</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>Release of H+ ions during ATP hydrolysis by ATPase associated with erythrocyte membranes prepared from normal calves and calves infected with <em>A. marginale</em></td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>Effect of membrane protein concentration on ATPase activity associated with erythrocyte membranes prepared from normal calves and calves infected with <em>A. marginale</em></td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>Effect of incubation time on ATPase activity associated with erythrocyte membranes prepared from normal calves and calves infected with <em>A. marginale</em></td>
<td>36</td>
</tr>
<tr>
<td>5</td>
<td>Effect of pH on total ATPase activity associated with erythrocyte membranes prepared from normal calves and calves infected with <em>A. marginale</em></td>
<td>37</td>
</tr>
<tr>
<td>6</td>
<td>Effect of temperature on total ATPase activity associated with erythrocyte membranes prepared from normal calves and calves infected with <em>A. marginale</em></td>
<td>38</td>
</tr>
<tr>
<td>7</td>
<td>Lineweaver-Burk plot for Km of ATP of total ATPase activity associated with erythrocyte membranes prepared from normal calves and calves infected with <em>A. marginale</em></td>
<td>56</td>
</tr>
<tr>
<td>8</td>
<td>ATPase activity associated with erythrocyte membranes prepared from 16 normal calves and 16 <em>A. marginale</em>-infected calves at peak infection</td>
<td>57</td>
</tr>
<tr>
<td>FIGURE</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>9</td>
<td>Ratio of sodium-potassium-magnesium dependent ATPase (Na,K-ATPase)</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>activity to magnesium-dependent ATPase (Mg-ATPase) activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>associated with erythrocyte membranes prepared from 16 normal calves</td>
<td></td>
</tr>
<tr>
<td></td>
<td>and 16 calves infected with <em>A. marginale</em> at peak infection ..........</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Total ATPase activity associated with erythrocyte membranes prepared</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>from three calves before and during the course of infection with</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>A. marginale</em></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Ouabain-insensitive ATPase activity associated with erythrocyte mem­</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>branes prepared from three calves before and during the course of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>infection with <em>A. marginale</em></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Ouabain-sensitive ATPase activity associated with erythrocyte mem­</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>branes prepared from three calves before and during the course of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>infection with <em>A. marginale</em></td>
<td></td>
</tr>
</tbody>
</table>
ABSTRACT

The ATPase (adenosine 5'-triphosphate phosphohydrolase EC 3.6.1.3.) activity of erythrocyte membranes from young calves was investigated before and after infection with Anaplasma marginale. A three-fold increase in the initial rate of ATPase was measured at the peak of infection in erythrocyte membranes prepared from calves infected with A. marginale when compared to erythrocyte membranes prepared from normal calves.

A magnesium-dependent ouabain-insensitive ATPase (Mg-ATPase) and a sodium-potassium-dependent ouabain-sensitive ATPase (Na,K-ATPase) associated with bovine erythrocyte membranes were confirmed. Both components of ATPase activity exhibited an absolute requirement for magnesium ions; therefore, this enzyme system possesses the characteristics of a transport ATPase.

Optimal conditions of incubation for maximal activation of the enzyme system were determined. The pH optimum, temperature optimum, substrate specificity, and ouabain concentration required for inhibition of activity were similar to those of the membrane ATPase of other mammalian erythrocytes. Temperature and pH optima did not change during anaplasmosis. Adenosine 5'-triphosphate (ATP) was preferentially hydrolyzed by the enzyme of membranes
prepared from normal erythrocytes and erythrocytes from calves parasitized with *A. marginale*. The enzyme prepared from erythrocytes from calves parasitized with *A. marginale* hydrolyzed inosine 5'-triphosphate (ITP) at a faster rate than did enzyme prepared from normal erythrocytes. Hydrolysis of other substrates was insignificant.

The concentration of ouabain required to completely abolish Na,K-ATPase activity associated with normal erythrocyte membranes was similar to reported values. However, the Na,K-ATPase activity associated with membranes of erythrocytes infected with *A. marginale* was abolished by ouabain at a concentration lower than that reported for the enzyme bound to normal erythrocyte membranes.

ATPase activity was sensitive to changes in the ratio of sodium:potassium ions. The optimum sodium:potassium ratio was 6:1.

An optimum magnesium:ATP concentration ratio of 1:1 and 0.5:1 was established for the Mg-ATPase and the Na,K-ATPase activities, respectively.

The Km for ATP of the enzyme system decreased significantly during anaplasmosis.

Total ATPase activity and Mg-ATPase activity increased significantly during the early stage of the infection at the initial appearance of *A. marginale* in erythrocytes and during the peak of the infection at the maximum per cent of parasitized erythrocytes observed. Na,K-ATPase activity did not change significantly during anaplasmosis infection. Therefore, the per cent of inhibition of total ATPase
activity was decreased during the disease since the increase in activity was due solely to a three-fold increase in Mg-ATPase activity. No changes in ATPase activity occurred when anemia was experimentally induced.

Significant Mg-ATPase activity was associated with partially-purified _A. marginale_.

Antiserums prepared against membranes from normal calves and calves infected with _A. marginale_ were incubated with various membrane preparations to compare their effect on ATPase activity. Significant ATPase activity in membranes from _A. marginale_-infected calves was measured which could be attributed to _A. marginale_.

xi
INTRODUCTION

Anaplasmosis (bovine infectious anemia) is an infectious and transmissible disease of ruminants, the severity of which varies from peracute to chronic (17, 18, 71, 73). Acute anaplasmosis is characterized by a severe, progressive anemia associated with the presence of intraerythrocytic inclusion bodies (113). **Anaplasma marginale**, the causative agent, is classified as a rickettsia (11), but its exact nature has been a continuing taxonomic problem (113). The structural and developmental resemblance between *A. marginale* and rickettsia and its close morphologic, serologic, and metabolic resemblance to *Eperythrozoon* and *Haemobartonella* have led to the classification of *A. marginale* under the order of **Rickettsiales** (52, 79, 111, 112, 133).

The marginal bodies are observed in blood smears stained with Wright's or Giemsa stain as small, rounded, basophilic bodies located in the stroma of the erythrocytes near their margins (140). Extraerythrocytic bodies stain Gram negative (1). Electron micrographs reveal these marginal bodies to range in diameter from 0.3 to 1.0 µ and are composed of 1-8 smaller initial bodies (59, 109, 115, 121). The initial body is thought to be the true etiologic agent, capable of invasion of the mature erythrocyte and multiplication by binary fission to form the mature marginal body (116).
Anaplasmosis is a major disease of economic importance in cattle throughout tropical and subtropical regions of the world (140). Due to the continuous widespread movement of cattle, the incidence of anaplasmosis in this country has steadily increased and thus economic loss has increased (119). Although most prevalent in the gulf and western states, the disease has been diagnosed in most states in the United States (140).

Anaplasmosis is chiefly a disease of cattle. Other ruminants, including sheep, goats, antelope, deer, and elk are less susceptible, but may serve as a reservoir of infection (20). Animals that survive initial infections become permanent carriers and serve as reservoirs (28). Non-ruminant domestic animals, laboratory animals, and man are not susceptible.

Early research efforts in the study of this disease were directed at etiology, taxonomy, and treatment. Immunological, pathological, and biochemical studies have been initiated in recent years. However, the inability to propagate the parasite in vitro has proven a hindrance to biochemical and metabolic studies. Therefore, research efforts have been principally concerned with changes in metabolic and biochemical patterns within the host.

At the peak of anaplasmosis infection, Garon and Dimopoullos (58) observed that erythrocyte ATPase (adenosine 5'-triphosphate phosphohydrolase EC 3.6.1.3) activity increased three-fold above that of normal erythrocytes. This increased activity was correlated with an increase in cation
permeability of the membrane. ATPase activity was also noted in fractions of partially-purified *A. marginale* bodies. Since ATPase activity is intimately associated with active transport and metabolism in erythrocytes, it was proposed that the production and/or utilization of energy of the erythrocytes was impaired as a result of the infection.

The purpose of this investigation was to further characterize the changes in the components of ATPase activity of the erythrocyte membrane and to correlate such changes with the pathogenesis of anemia in anaplasmosis.
A. Historical Aspects

Smith and Kilborne (136) first described marginal bodies in erythrocytes of cattle. However, they believed that these bodies were a developmental stage in the life cycle of *Piroplasma bigemina*, the causative agent of piroplasmosis. Theiler (141) considered these bodies to be a new genus to which he gave the name *Anaplasma marginale* because of their apparent lack of cytoplasm and marginal location within the erythrocytes. Thus, the disease became known as anaplasmosis. Following the simultaneous eradication of *Boophilus* ticks and piroplasmosis, anaplasmosis was recognized as a disease (24). The first case in the United States was recorded in 1913 (14). The disease has been reported throughout the tropical, subtropical, and temperate regions of the world and in most states in the United States (108). Due to the continuous widespread movement of cattle, the incidence of anaplasmosis in this country has steadily increased until the infection rate in the United States is estimated at six per cent of all adult animals tested (119). The mortality rate has been reported to range from ten to ninety per cent (42). Consequently, anaplasmosis is a disease of major economic importance to the cattle industry, causing about $100,000,000 in losses annually (155).
B. Taxonomical Aspects

Anaplasma marginale is classified in the order Rickettsiales and the family Anaplasmataceae (11). However, reported morphologic evidence suggests classifying the organism as a virus (51), a protozoon (16, 27, 46, 47, 53, 59), somewhere between viruses and rickettsia (108), as well as a rickettsial organism (1, 79, 86, 108, 121). Inability of A. marginale to grow in artificial culture media and its requirement for an intracellular environment for multiplication suggests its metabolic requirements may be similar to those of rickettsia (115). Further similarity between A. marginale and rickettsia is indicated by common staining characteristics, transmission by arthropods (29), interference of A. marginale with development of infection with the rickettsial organism Eperythrozoon (52), sensitivity to broad spectrum antibiotics (87), and propagation in cells such as erythrocytes which have a low rate of metabolism (109). Moreover, A. marginale possesses common antigens with rickettsial organisms (112) and Anaplasma marginal bodies appear to be structurally similar to Eperythrozoon and Haemobartonella (79, 133). However, A. marginale has been shown to share common antigens with several plasmodial species (32). However, unlike Plasmodium and Babesia, A. marginale does not deoxygenate hemoglobin (104). The respiration rate of A. marginale is significantly lower than that of typical blood protozoon parasites and approximates that of rickettsia (104, 111). Therefore, it is evident that A. marginale has not been unequivocally taxonomically classified.
C. **Clinical Aspects**

The major clinical symptoms of anaplasmosis include labored respiration, icterus, elevated body temperature, weakness, dehydration, and anorexia (17, 18, 71, 73). Dairy animals recovering from anaplasmosis produced 25 per cent less milk and 30 per cent less milkfat during the concurrent lactation period (119). These same animals averaged seven per cent lower milk production in subsequent lactations than did non-infected animals in the same herd and remained in the herd approximately one year less (119).

The severity of symptoms in anaplasmosis varies considerably with age (71, 72, 73). Calves to one year of age usually undergo mild, subclinical infections with little or no mortality (72). The disease is more severe in cattle between the ages of one and two years, but recovery is the rule (72). In cattle two to three years of age, anaplasmosis is acute and often fatal (72). Anaplasmosis is peracute and usually fatal in animals over three years of age (72). As the disease progresses, older animals exhibit irrational behavior, become belligerent and excited at the slightest provocation, and may die of asphyxia (140). Abortion is common in advanced pregnancy (140).

The course of clinical anaplasmosis may be only a day in the fatal form, 14 to 21 days in the typical acute and subacute form, or even longer in the occasional chronic form (72). Animals surviving the period of erythrocyte destruction will usually recover gradually or remain emaciated and unproductive (140). Animals which survive the
disease usually remain permanent carriers (140). At least 17 species of ticks and 9 species of horseflies are capable of transmitting anaplasmosis (14, 28, 156).

D. Biological Aspects

Recent studies of some of the biochemical properties of purified A. marginale have been fruitful in determination of the metabolic patterns of the parasite (31). A. marginale has been shown to possess lactate dehydrogenase (25), catalase (146), and adenosine triphosphatase (58). It has been suggested that ATPase activity affects the energy requirements of the erythrocyte and the movement of cations across the erythrocyte membrane (57, 58). However, no change in cation concentrations during anaplasmosis has been detected (57). The increase in ATPase activity, however, may be related to the increase in cation permeability of erythrocyte membranes (57). A. marginale has been shown to inhibit erythrocytic acetylcholinesterase, which further increases permeability of the membranes (145). Significant changes in the fatty acid profile of extracted lipid from erythrocytes of cattle infected with A. marginale and a substantial decrease in phospholipid of erythrocyte membranes may contribute to increased cation permeability and the mechanism of anemia (36, 122). An increase in sialic acid content and electrophoretic mobility of erythrocytes infected with A. marginale was observed (37). Research has confirmed erythrocytes infected with A. marginale undergo ultrastructural membrane changes which increase osmotic fragility and
contribute to the progressive anemic state of the disease (33, 34).

There is considerable evidence that anaplasmosis provokes an autohemolytic immune response in the host (15, 23, 84, 126, 127). It has been established that the degree of anemia is not proportional to the degree of parasitemia (114). Both erythrocyte-bound and free-serum antibodies have been detected which were serologically distinct from those detected in various diagnostic tests (84, 126). A heat-stable opsonin for autologous and homologous erythrocytes in the serum of calves infected with *A. marginale* that sensitized normal erythrocytes to phagocytosis in vitro has been demonstrated (127). Opsonin titers coincided with the intensity and persistence of anemia in calves. An autoantibody–erythrocyte complex in vivo in diseased calves was reported (23). Recently a migration-inhibition factor from leukocyte cultures from carrier calves has been identified, and evidence for a cell-mediated immune response in anaplasmosis has been reported (15). The autoimmune syndrome may contribute to the removal of damaged erythrocytes from the peripheral circulation by the reticuloendothelial system and the resulting hemolytic anemia (110).

In the last two decades, the erythrocyte has been shown to be more than an inactive mechanical package with the sole purpose of transporting hemoglobin. The erythrocyte is one of nature's ultimate examples of a harmonious blending of structure and function. The mature erythrocyte is
incapable of synthesizing hemoglobin, membrane protein, or metabolic enzymes required to support its primary role—delivery of oxygen to the tissues from the lungs and the transport of carbon dioxide from the tissues to the lungs. However, erythrocytes are extremely metabolically active, and this is essential for their survival. Energy must be provided to maintain cation gradients, to generate reduced nicotinamide adenine dinucleotide (NADH) which serves as a cofactor to reduce the hemoglobin molecule, to protect proteins against oxidative denaturation, and to maintain its shape (12, 56, 106, 151). Since the mature erythrocyte lacks a nucleus, mitochondria, and endoplasmic reticulum, it must use the relatively inefficient Embden-Meyerhof pathway for adenosine 5'-triphosphate (ATP) production and the hexose monophosphate pathway for a source of reducing power (12).

A massive proliferation of knowledge concerning the function and metabolism of erythrocytes has resulted in the recognition of defects of membrane structure and function, abnormalities of hemoglobin synthesis or structure, and deficiencies of enzymes, all of which may result in hemolytic anemia due to the shortened survival of the erythrocytes. These abnormalities have provided a greater understanding of normal erythrocyte metabolism and the mechanisms of cell destruction.

Correlation between mean age of the erythrocyte and enzymatic activity has been made in man and other animals (135). Each enzyme has a characteristic decay rate, therefore,
shortened mean erythrocyte lifespan may follow from any cause of increased enzymatic activity. Activity of bovine erythrocyte enzymes decrease as the cell ages, but not to the same degree as those of human erythrocytes (135). Glucose-6-phosphate dehydrogenase and lactate dehydrogenase increase significantly in horse erythrocytes during equine infectious anemia (75). In anemic cattle, glutathione reductase and pyruvate kinase are significantly elevated, but lactate dehydrogenase is not (135). The most marked change in the activity of the above enzymes associated with age of bovine erythrocytes was during the erythropoietic response (135).

The erythrocyte is commonly employed as a model system for the study of mammalian cells. Because of its ease of collection, its lack of subcellular organelles, and its relatively simple cytoplasmic constituents, the erythrocyte membrane is easily isolated. Consequently, erythrocyte membranes have become a model system for the study of membrane structure and function. Furthermore, it is an ideal source for the study of membrane-bound enzymes and their involvement in cellular metabolism.

With increased interest in active transport of cations it was only natural that research in this area would turn to the erythrocyte. Consequently, the human erythrocyte membrane has become one of the best studied systems with regard to the characteristics and mechanism of active cation transport (149). In the past decade considerable evidence has been accumulated to support the concept that ATPases are intimately involved in the active transport of cations.
across biologic membranes (30, 38, 76, 131). After demonstration of an enzymatic activity in crab nerves which was dependent upon the presence of sodium and potassium for maximal activation (130), correlation between intracellular location of this enzymatic activity and active transport systems in a variety of tissues was made (30, 38, 67, 76, 131, 142). It was not detected in tissues without cells or in tissues with a very low cell density. In all tissues ATPases are membrane-associated enzymes.

Mammalian erythrocytes possess two or more types of ATPase activity associated with both the intact erythrocyte and the isolated erythrocyte membrane (5, 63, 105). All types utilize ATP as an energy source and hydrolyze one of the high energy phosphate groups from ATP. For activity, all types have an absolute requirement for magnesium (130, 134). One type (Na,K-ATPase), which requires sodium and potassium for stimulation, is inhibited by cardiac glycosides, and maintains, by means of active transport, high levels of potassium and low levels of sodium within the erythrocyte (63, 130). The other types (Mg-ATPase or Ca-ATPase), which do not require sodium or potassium for stimulation, are insensitive to cardiac glycosides, and may not be involved in cation transport (5, 105, 149).

There is wide disagreement on the substrate specificity of ATPase. In addition to ATP, there is evidence that inosine 5'-triphosphate (ITP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP), and adenosine 5'-diphosphate (ADP) may be hydrolyzed by erythrocyte ATPases (153).
In erythrocytes from many species, the ATPases all had the above mentioned characteristics (67, 131). However, quantitative differences in the pH optimum, in the affinities for sodium and potassium, and in the Na,K-ATPase:Mg-ATPase activity ratio were observed. Inhibition by cardiac glycosides was not a uniform property of cation-stimulated ATPase from all sources. Both cationic specificity and sensitivity to cardiac glycosides may vary with the source of the enzyme (142, 160).

In addition to involvement with active transport, erythrocyte ATPase serves an important role in the metabolism of erythrocytes (78, 93, 153, 161). The membrane cation pump is linked to glycolysis through the production of ADP by ATPase and the ADP produced by membrane ATPase contributes to the requirements for ADP in the cell (12, 44). Glycolysis and lactate production by erythrocytes was reduced when the ATPase pump was inhibited by ouabain (161).

Maintenance of biconcave shape and cation transport, the two essential energy-requiring functions of the erythrocyte, may be characterized as two processes competing for the utilization of ATP (56). Ouabain inhibits cation transport, but has no effect on erythrocyte morphology (56).

Evidence has been obtained that the function of ATPase is dependent upon: the degree of glutathione reduction, proper lipid environment, supply of ATP, rate of removal of ADP, and the supply of NADH (30).

The level of Na,K-dependent ATPase was found to be considerably higher in reticulocytes than in mature erythrocytes.
Higher activity in reticulocytes is necessary for the active transport of amino acids which is linked to cation transport (153, 160). As the cells mature, protein synthesis decreases. Inability to concentrate amino acids occurs concomitantly with the decrease in ATPase activity.

In human infants, ouabain-sensitive ATPase is significantly less than in adults, but the total ATPase activity is significantly greater (148). Enzyme activity is at the highest in the neonate and in children below the age of three. This activity decreases with age until puberty at which point the level of activity normally is maintained for life (129).

In most species, the Na,K-ATPase activity is related to the intraerythrocytic cation concentration (77). Dog and cat erythrocytes contain low potassium and high sodium levels and seem to lack a Na,K-ATPase (21). Erythrocytes of man and rat contain high potassium and low sodium levels and have substantial Na,K-ATPase activities (21). Intraerythrocytic cation concentrations in bovine are intermediate between those of man and dog (74). The presence of a Na,K-ATPase in the mature erythrocytes of cattle has been both affirmed (58, 77, 82) and denied (64, 65). However, as in the case of young humans, calves have high potassium erythrocytes and high Na,K-ATPase activity (13, 64). Potassium content and ATPase activity decrease as the cattle mature. All mature Holstein cattle are reported to have a uniformly low potassium intraerythrocytic concentration (13, 65).
In sheep and opossums two genetic types of erythrocytes have been observed (3, 40, 143). The Na,K-ATPase is four-fold more active in the high intraerythrocytic potassium (HK) type than in the low intraerythrocytic potassium (LK) type (66, 143). However, until maturity, all lamb erythrocytes are of the HK type and demonstrate high ATPase activity (143). There is evidence that Jersey cattle erythrocytes may exhibit a polymorphism in their potassium concentrations (45).

Many methods for assay of erythrocyte-bound ATPase have been reported (35, 105). The chemical and physical treatment employed to prepare erythrocyte membranes for assay of ATPase influenced the activity of the enzyme (2, 9, 64). Consequently, ATPase activity may reflect structural, chemical, or conformational change in the erythrocytic membrane (10, 63, 137). Short-term sonication of erythrocyte membranes is reported to increase both total ATPase and Na,K-ATPase activity (64). That activity of ATPase, which is stimulated by either sodium or potassium, is enhanced and is no longer sensitive to ouabain after sonication (2, 157). Sodium dodecyl sulfate effects a two-fold increase in ATPase activity stimulated by either sodium or potassium and sensitivity to ouabain is lost (19). These results do not suggest assay of more than one enzyme, but different manifestations of the enzyme induced by physical or chemical treatment during preparation (2). Shearing of erythrocyte membranes in a French pressure cell decreases the activity of both types of ATPase without altering lipid content,
sulfhydryl group reactivity, or sialic acid content and orientation (123, 125).

Freezing of erythrocyte membranes increases total ATPase and Na,K-ATPase (120) or Mg-ATPase (157) considerably. This enhanced activity increases with decreasing temperature and time held in the frozen state. The rate of cooling is a more effective factor than the final temperature; the faster the rate of cooling, the greater the increase in ATPase activity (137). The increased activity appeared attributable to increased accessibility of ATP to the enzyme due to disruption in the erythrocyte membrane (137).

Preincubation of erythrocyte membranes with digitonin increases the Mg-ATPase activity (157). Mg-ATPase is markedly decreased by aging of erythrocyte ghosts in various buffers (68).

Hanahan and Ekholm (64) observed that during the preparation of human erythrocyte membranes by hypotonic lysis, there is a decrease in total ATPase activity and concomitantly, a progressive alteration of the membranes.

These changes are pronounced at pH 7.6 and occur to a lesser extent at pH 5.8. General permeability and integrity of the membranes were greatly impaired at low osmolarities (78). Ca-ATPase activity is lost completely, but effects were lessened if membranes were stored in isotonic buffers (78). Examination of calf and goat erythrocytes showed that their membranes are maximally altered in the first hypotonic wash at pH 7.6 (64). Again a loss of total ATPase activity and protein was noted. However, total ATPase activity of
erythrocyte membranes increases with decreasing osmolarity of the buffer employed for hypotonic lysis (10). The ouabain-sensitive component of ATPase increased four-fold by decreasing the electrolyte concentration from 325 to 25 mM (22). Low ionic strength has deleterious effects upon the morphological integrity of human erythrocyte ghosts by increasing fragmentation (9). These effects accompany the loss of non-hemoglobin protein from the membranes. The presence of calcium, and to a lesser extent magnesium, protects against some of the effects of low ionic strength (9). Calcium-prepared membranes were found to be less permeable than those prepared with EDTA (9). These results emphasize some of the problems encountered in correct interpretation of the characteristics of erythrocyte membranes and point out that the Na,K-ATPase activity appears to have more stringent organizational or structural requirements than any of the partial reactions in erythrocyte membranes (2, 62, 123, 124, 125, 158).

Solubilization of the erythrocyte membrane has resulted in irreversible loss of both Mg-ATPase and Na,K-ATPase (123, 125). The activation or inhibition of ATPase activity by inorganic substances has been characterized in the erythrocyte. ATP hydrolysis is highly dependent upon the relative concentrations of potassium, sodium, magnesium, and calcium ions (65, 130, 132, 134). In the complete absence of alkaline-earth metals, no ATP hydrolysis occurs (130). The optimum magnesium:ATP ratio is 1:1 or less (132, 134). Potassium can be replaced by ammonium, rubidium, cesium, and lithium (in
order of decreasing efficiency) in stimulating ATPase activity (76). Na,K-ATPase exhibits absolute specificity for sodium (76, 107).

The mode of action of several inhibitors, notably cardiac glycosides, upon erythrocyte ATPase and active transport has been characterized. Ouabain specifically inhibits active transport by inhibition of the Na,K-ATPase of erythrocytes (38, 68, 124). However, ouabain inhibits total ATPase activity in rabbit erythrocyte membranes which contain no detectable Na,K-ATPase (55). This effect may be due to membrane binding of sodium and potassium which has been shown to occur with human erythrocyte ghosts (118). HK type cells of sheep require six times more ouabain molecules bound per cell than do LK type cells to effect complete inhibition of Na,K-ATPase (40). Ouabain inhibition is markedly influenced by the concentrations of magnesium, sodium, potassium, and ATP in the medium (132). No binding of ouabain is observed in the absence of ATP since ouabain binds to one of the phosphorylated ATPase intermediates (159). The effects of ouabain can be reversed by the addition of excess potassium to the medium (159). Ouabain-potassium antagonism exhibits kinetics of a competitive type of inhibition (159).

Both the rate and steady-state level of ouabain inhibition is increased with increasing levels of magnesium not complexed with ATP (132). Although the association rates of ouabain with different tissues and species are all similar, their dissociation rates may vary up to 60 fold (142).
In human erythrocyte membranes, radioactively-labelled ouabain binds to a partially-purified Na,K-ATPase protein fraction (39). Schrier et al (124) have shown that ouabain inhibition is more effective in less rigorously prepared membranes. Ouabain inhibition of ATPase was complete in erythrocyte membranes which had not been completely washed free of hemoglobin, and was incomplete and not predictable in hemoglobin-free erythrocyte membrane preparations. The latter authors suggested that a ouabain-responsive site was impaired in washed erythrocyte membranes. Loss of ATPase inhibition by ouabain was not correlated with a loss of sodium and potassium stimulation (124).

In addition to cardiac glycosides, a number of other organic inhibitors have been employed to study erythrocytic ATPase. Sulfhydryl blocking agents, such as N-ethylmaleimide (NEM) and p-chloromercuribenzoate, inhibit total erythrocyte ATPase activity (6, 60, 134). In some cases, NEM mimics cardiac glycosides and preferentially inhibits the Na,K-ATPase activity (63). Delta-aminolevulinic acid has no effect upon the Na,K-ATPase activity of erythrocyte membranes, but it does inhibit Na,K-ATPase of intact erythrocytes (4).

The use of oligomycin as an ATPase inhibitor led to the proposal of a sodium-sensitive site and a potassium-sensitive site on the enzyme (78). Oligomycin blocks stimulation of ATPase by sodium but not potassium-dependent ATPase activity, whereas ouabain inhibits both (78). An oligomycin binding site can be blocked by shearing erythrocyte membranes.
without altering the sodium and potassium stimulation of ATPase (123).

The role of chemical composition and structure of erythrocyte membranes in the functioning of ATPase is not well understood in spite of extensive studies. Erythrocyte ATPase is usually referred to as an enzyme, but is probably not composed of protein alone since lipids have been shown to be intimately involved as a component of the catalytic unit (150, 153).

Incubation of membrane preparations with proteases causes a progressive loss of enzymic activity (63). The role of lipids in ATPase function is unclear (153). ATPase is found in erythrocyte membranes of considerably different lipid composition although the general properties of the ATPases are similar. Solubilization of the membranes with sodium deoxycholate or saponin results in complete loss of ATPase activity (150). Enzymatic activity is not restored after removal of the detergent by dialysis but is restored by the addition of phospholipids (150). Extraction of lyophilized erythrocyte membranes with ether has no effect on Na,K-ATPase activity although all the sterols and 23 percent of the phospholipids are removed (117, 149). All ATPase activity is abolished by further extraction of lipid components with petroleum ether or n-butanol and cannot be restored by the addition of phospholipids (149). ATPase activity is partially inhibited by phospholipase A or C (117, 139) and may be restored by the addition of phospholipids (49). Ouabain binding and inhibition is not decreased
significantly by treatment with phospholipase (138). Data obtained after shearing of erythrocyte membranes into microvesicles of varying size, structure, and chemical composition has shown that Na,K-ATPase is associated with fractions relatively rich in trilamellar membrane structures (123). These results and others (62) lead to the conclusion that preservation of lipoprotein structure and not necessarily gross membrane structure is most important in preserving ATPase activity and that an intact phospholipid bilayer is necessary for ATPase activity.

Studies of sheep erythrocyte lipids have shown that there was no quantitative differences between HK and LK erythrocyte types in the distribution of individual lipid classes, phospholipid classes, total fatty acid composition, or component fatty acids of phospholipids (98, 99, 100). Therefore, it appears that structural differences in the membrane as a whole were probably more important than specific enzyme concentrations in the cell. This was supported by evidence that ATP levels are higher, rather than lower, in HK type cells (43), metabolism of the cells appears to be similar (99), and the velocity of Na,K-dependent ATP hydrolysis and ouabain binding in LK type membranes is markedly increased after treatment of the membranes with antiserum (7). After treatment with LK antiserum the Na,K-dependent ATPase activity of LK type membranes approaches that of HK type membranes without treatment.

It appears from the foregoing that ATPase activity is intimately associated with erythrocyte membrane structure.
and function and changes in ATPase activity may be correlated to changes in protein conformation and integrity of the membrane.
MATERIALS AND METHODS

A. Experimental Animals

Calves of mixed breed from 2 weeks to 3 months of age were procured from local auction sales or dairies. These animals were maintained on grain rations and given hay and water free choice. Splenectomies were performed prior to experimentation. Experimental clinical cases of anaplasmosis were induced by intravenous inoculation of 50 ml of whole blood from an animal with an acute infection. Uninoculated, splenectomized calves served as controls and were maintained similarly.

B. Hematology

Blood samples for hematologic examination were obtained by jugular phlebopuncture into evacuated glass containers (16 x 100 mm) which contained 12 mg disodium ethylenediamine-tetraacetic acid (EDTA) as an anticoagulant. These samples were examined for per cent of parasitized erythrocytes and packed cell volumes (PCV). Blood smears were prepared on glass slides, fixed with methanol for 2 minutes and stained with Giemsa stain for 20-30 minutes. Basophilic Anaplasma bodies were observed near the periphery of intact erythrocytes using the oil immersion lens (mag. 900 x) of a light microscope. Per cent of parasitized erythrocytes was calculated by counting the number of parasitized and non-parasitized erythrocytes in several fields. Packed cell
volumes were determined by the microhematocrit method.

Blood samples were categorized according to the clinical stage of the infection when procured as follows:
(1) early—1 to 5 per cent of erythrocytes parasitized and no significant change in PCV from normal; (2) middle—5 to 35 per cent of erythrocytes parasitized and 1 to 5 per cent decrease in PCV from normal; (3) late—29 to 55 per cent of erythrocytes parasitized and 5 to 15 per cent decrease in PCV from normal; (4) peak—56 per cent or more of erythrocytes parasitized and a PCV of 15 per cent or more below normal. When infection attained a desired clinical stage, blood was obtained aseptically by: (1) jugular phlebotomy when small volumes (500 ml or less) were required; or (2) exsanguination by cardiopuncture when larger samples were required. Heparin was used as an anticoagulant.

C. Experimental Induction of Anemia

In order to compare the secondary role of anemia to the effects of *A. marginale* infection, two types of experimental anemias were produced. These anemias were comparable in severity to the anemia caused by *A. marginale* infection. A severe anemia was produced in one experimental animal by periodic dosages of phenylhydrazine hydrochloride in 20 ml of isotonic saline solution as follows: A 1.0 gm dose was given each day for the first 2 days, 1.5 gm on the third day, and 2.0 gm on the fourth day. In another animal a severe anemia was produced by jugular phlebotomy on four successive days. One-half liter of whole blood was let on
the first and second day and three-fourth liter on the third and fourth day.

D. Preparation of Materials

1. Erythrocyte membranes

Heparinized whole blood was centrifuged for 20 minutes at 1080 x g and 4 C in a Sorvall RC-2 centrifuge using a GSA or SS-34 rotor. Plasma, buffy coat, and the upper one-fourth of the packed erythrocytes were removed by aspiration. The remaining cells were washed three times in 0.002 M Tris-HCl, 0.15 M NaCl, pH 7.4 cooled to 4 C. Removal of buffy coat was repeated by careful aspiration after each centrifugation.

Washed red blood cells were hemolyzed with 10 volumes of a hypotonic solution (0.005 M CaCl₂, 0.005 M Tris-HCl, pH 7.8, 4 C) and centrifuged at 27,000 x g at 4 C for 20 minutes. The supernatant fluid was decanted and the sedimented membranes were washed repeatedly with the above solution until free of hemoglobin. These fluffy membranes, white or light pink, were suspended in an equal volume of hemolyzing solution. The protein concentration of the membrane suspensions was determined in quadruplicate by a colorimetric method (61) with bovine serum albumin as a standard. Suspensions of erythrocyte membranes were diluted to a desired concentration in the hemolysis solution described above and stored at 2 C for immediate use (within a week) or -76 C for future use.
An alternate method of preparation of erythrocyte membranes was followed in some cases. Erythrocytes were washed as above and alternately frozen at -76 C and thawed at 37.5 C three times. After each freeze-thaw cycle, membranes were centrifuged and resuspended in fresh washing solution. Protein determination and storage followed as described above.

2. Marginal bodies

Heparinized whole normal blood and blood from calves infected with *A. marginale* was centrifuged and washed as described for erythrocyte membranes. Packed cells held at 4 C were sonicated using a Model LS75 Branson Sonifier at 8 amperes for 90 seconds. Intact erythrocytes and cellular debris were removed by centrifugation at 1080 x g at 4 C for 10 minutes. Supernatant fluid was collected and centrifuged at 27,000 x g at 4 C for 30 minutes. The resulting crude marginal body preparation was washed with buffer held at 4 C until free of hemoglobin. Blood from a normal calf was treated similarly and served as a control.

One portion of the marginal body preparation or control sample was employed immediately in an assay for ATPase activity. Another portion was purified further by sucrose density gradient centrifugation techniques. Non-linear gradients were prepared by layering 4, 7, 7, and 7 ml of a solution containing 60, 50, 40, and 30 per cent sucrose (w/v), respectively, in cellulose nitrate tubes. Linear gradients were prepared using a gradient mixer and solutions of 60 and 30 per cent sucrose as described by Martin and
Ames (85). A 3 ml aliquot of the marginal body preparation was carefully layered over the gradient, and the tubes centrifuged at 50,000 x g at 4 C for 90 minutes using a Spinco Model L Ultracentrifuge and an SW-25.1 swinging bucket rotor. The layers were carefully separated, washed free of sucrose solution with hemolysis buffer, and employed immediately for an assay for ATPase activity.

3. Anti-erythrocytic membrane serum

Antiserum to pooled samples of normal erythrocyte membranes (anti-N) and infected erythrocyte membranes (anti-I) were produced in New Zealand white rabbits. Normal rabbits were used as a source of non-immune (NRS) serum.

E. Measurement of ATPase Activity

1. General

The rate of hydrolysis of ATP in the presence of various preparations was measured by following the rate of release of either orthophosphate (Pi) or H+ ions. The protein concentration of the membrane preparation, final concentrations of various salt solutions, inhibitors and substrates, pH, temperature, and time of incubation were carefully controlled. All salt solutions were prepared from the chloride salts of magnesium, sodium, calcium, and potassium. Generally, each assay was made in triplicate. Each experiment was repeated with erythrocyte membranes prepared from three animals.

2. Standard assay

The standard assay used for determination of total
ATPase activity was conducted at pH 7.8 and 37.5 C for 60 minutes in a solution containing final concentrations of 0.6 mg/ml or less of membrane protein, 3 mM Tris-ATP or disodium ATP, 3 mM MgCl₂, 120 mM NaCl, 20 mM KCl, and 40 mM Tris-HCl buffer. Ouabain-insensitive ATPase was determined by the inclusion of 0.5 mM ouabain octahydrate to the standard assay mixture. Mg-ATPase activity was measured by the exclusion of NaCl and KCl salts. Na,K-ATPase was determined by subtraction of Mg-ATPase or ouabain-insensitive ATPase from the total measured ATPase activity.

3. Initial rate of ATPase

The initial rate of ATPase of normal erythrocyte membranes and preparations from calves infected with *A. marginale* was determined by quantitation of H⁺ ions released into a standard unbuffered (40 mM Tris-HCl excluded) assay mixture. The reaction vessel was placed in a water bath at 37.5 C, and 4.9 ml of assay medium containing membranes was added. The mixture was stirred continuously with a rubber-tipped glass rod. pH was measured with a combination glass-silver chloride electrode and a Beckman Expandomatic pH meter in the expanded mode. pH changes were recorded on a Beckman six-inch recorder with a span of 1 pH unit. When temperature equilibration was achieved, the reaction was initiated by the addition of 0.1 ml of 150 mM ATP (previously buffered to pH 7.8 with 1.0 N and 0.1 N NaOH). The reaction was recorded sufficiently long enough for accurate measurements (usually one minute) or until the recorded slope was
no longer linear due to change in pH of the assay medium. Reaction rates were expressed as increase in nmoles H+/mg membrane protein/min.

4. Assay of ATPase

The activities of ATPase of normal blood membranes, membranes from calves infected with *A. marginale*, and marginal body preparations were determined by the quantitation of orthophosphate (Pi) released into the standard assay mixture with or without ouabain or salts added. Optimum conditions for total ATPase activity in normal erythrocyte membranes and membrane preparations from calves infected with *A. marginale* were determined experimentally. Various experiments were conducted, each with one variable (e.g., final concentrations of salts, substrates, inhibitors, membrane protein, and time of incubation, temperature, and pH).

The reaction was initiated by adding substrate (ATP) to the incubation mixture. Samples were taken at the beginning and the end of the incubation period. Assays conducted with boiled membrane preparations (80°C for 15 minutes), no membrane preparation, or no ions present served as controls. Samples were immediately mixed with trichloroacetic acid at 4°C to a final concentration of 8 per cent (w/v). After centrifugation at 1000 x g for 5 minutes, the supernatant fluid was analyzed for inorganic phosphate by a modification of the Berenblum and Chain method (83). Three ml of supernatant fluid was added to a test tube. One-half
ml of 5.0 M H$_2$SO$_4$, 5.0 ml of n-butanol and benzene (v/v), and 0.5 ml of 10 per cent ammonium molybdate were added sequentially. This mixture was shaken 15 seconds to extract the phosphomolybdate complex into the non-polar phase. After separation of the two layers, a suitable amount of the non-polar phase (0.1-2.5 ml) was pipetted into another tube. This aliquot was diluted to 5.0 ml with 3.2 per cent H$_2$SO$_4$ in absolute ethanol (v/v). One-half ml of 10 per cent SnCl$_2$·2H$_2$O in concentrated HCl freshly diluted 200 times with 0.5 M H$_2$SO$_4$ was then added and mixed immediately. Color intensity was measured at 630 nm with a Beckman DB Spectrophotometer and compared to standard phosphate samples. Activities were calculated and expressed as nmoles of Pi released/mg of membrane protein/hr. The difference in concentration of inorganic phosphorus between the zero-time sample or the various controls and the terminal sample was employed to calculate the amount of Pi released.

F. Precautionary Measures

Glass-distilled and demineralized water was used exclusively throughout these experiments. All glassware was cleaned with a non-ionic detergent (Acationox) and rinsed thoroughly with demineralized water before use. Reagents and standards were stored in glass stoppered Pyrex glassware or other appropriate containers at proper temperatures. Suitable standard buffers were used to calibrate the pH electrode and meter daily. The pH
electrode was rinsed with demineralized water and dried with disposable wipers after each use.
RESULTS

Preliminary experiments were conducted to measure the rate at which H+ ions were released during hydrolysis of ATP by ATPase associated with erythrocyte membranes from calves parasitized with *A. marginale*. Figure 1 records graphically data that show this rate for 60 seconds was linear at membrane protein concentrations up to 0.6 mg/ml. Similar data were obtained with normal erythrocyte membranes. In most cases, after the initial minute of incubation, the rate of release of H+ ions decreased due to the decrease in pH of the unbuffered assay medium.

A comparison of the average initial rate of total ATPase of erythrocyte membranes isolated from normal calves and calves infected with *A. marginale* is given in Figure 2. Results of this experiment showed that total ATPase activity increased approximately three-fold when calves are infected with *A. marginale*. No activity was detected in plasma of normal calves or calves infected with *A. marginale*.

However, this electrochemical method was not amenable to further characterization of the components of ATPase activity due to its sensitivity to minimal changes in pH when ATPase activators and inhibitors were included in the assay mixture. Subsequent assay of ATPase measured the release of orthophosphate into a standard buffered solution.
Fig. 1. Release of H+ ions during ATP hydrolysis by ATPase associated with erythrocyte membranes prepared from calves infected with A. marginale at different concentrations of membrane protein (● = 0.6 mg/ml, ○ = 0.3 mg/ml, O = 0.2 mg/ml).
Fig. 2. Release of $H^+$ ions during ATP hydrolysis by ATPase associated with erythrocyte membranes prepared from normal (●) calves and calves infected with *A. marginale* (○).
The relationships between ATPase activity, membrane concentration, incubation time, pH, and temperature were studied to define optimal conditions for activity of this enzyme system. Data presented in Figure 3 shows that ATPase activity of normal erythrocyte membranes and erythrocyte membranes from calves infected with *A. marginale* was proportional to a membrane protein concentration up to approximately 0.6 mg protein/ml of assay mixture in the presence and absence of ouabain. Accordingly, subsequent experiments always employed 0.6 mg membrane/protein ml or less. Dry weight of membrane preparations was not employed in this study since the lipid composition of the erythrocyte membrane varies when animals are infected with *A. marginale* (36, 122). The author has assumed that protein composition of erythrocyte membranes does not change during anaplasmosis.

The ATPase associated with normal erythrocyte membranes and erythrocyte membranes from diseased calves exhibited a maximum velocity up to one hour of incubation in the presence and absence of ouabain (Fig. 4). Maximal activity of the enzyme occurred at pH 7.8-8.0 at 35-40 C (Fig. 5, 6). Similar data were obtained when ouabain was included in the assay. Hence, one hour of incubation at pH 7.8 and 37.5 C was established as optimal for assay of ATPase activity.

The specificity of this enzyme system was examined by substituting ITP, adenosine 5'-monophosphate (AMP), and pyrophosphate for ATP and measuring the amount of orthophosphate liberated under identical conditions for one hour at 37.5 C and pH 7.8. The average amount of Pi liberated by
Fig. 3. Effect of membrane protein concentration on ATPase activity (— = total, — = ouabain-insensitive, --- = ouabain-sensitive) associated with erythrocyte membranes prepared from normal (○) calves and calves infected with *A. marginale* (●).
Fig. 4. Effect of incubation time on ATPase activity (— = total, —— = ouabain-insensitive, ••• = ouabain-sensitive) associated with erythrocyte membranes prepared from normal (○) calves and calves infected with *A. marginale* (●).
Fig. 5. Effect of pH on total ATPase activity associated with erythrocyte membranes prepared from normal (○) calves and calves infected with *A. marginale* (●).
Fig. 6. Effect of temperature on total ATPase activity associated with erythrocyte membranes prepared from normal (O) calves and calves infected with *A. marginale* (●).
each substrate in three separate experiments has been expressed as a percentage of the amount of ATP hydrolyzed by ATPase at optimal conditions with respect to sodium, potassium, and magnesium ions by erythrocyte membranes prepared from normal calves (Table 1) and erythrocyte membranes prepared from calves infected with \textit{A. marginale} (Table 2).

When erythrocyte membranes prepared from normal calves were the source of enzyme, 37 per cent of the ATPase activity was inhibited by ouabain, whereas only 19 per cent of ATPase activity was inhibited when erythrocyte membranes from calves infected with \textit{A. marginale} were the enzyme source. Hydrolysis of ADP was significant with erythrocyte membrane preparations. Inhibition of ADP hydrolysis by ouabain was similar in erythrocyte membrane preparations from normal and infected calves, approximately 26 and 34 per cent, respectively. AMP and pyrophosphate were hydrolyzed slowly compared with ATP under these conditions. Hydrolysis of AMP was not inhibited by ouabain when normal erythrocyte membranes provided enzyme. However, when erythrocyte membranes prepared from calves infected with \textit{A. marginale} provided enzyme, hydrolysis of AMP was slightly increased by ouabain. ITP hydrolysis was low in assays with normal erythrocyte membranes and significantly increased in assays made with erythrocyte membranes from diseased calves. With erythrocyte membranes isolated from normal and diseased calves, ITP hydrolysis was decreased approximately 50 per cent when ouabain was included in the assay mixture.
Table 1. Substrate specificity of ATPase associated with erythrocyte membranes from normal calves.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Assay medium</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I a</td>
<td>II b</td>
</tr>
<tr>
<td></td>
<td>(Total)</td>
<td></td>
<td>(Ouabain-insensitive)</td>
</tr>
<tr>
<td>ATP</td>
<td>100 c</td>
<td>63</td>
<td>37</td>
</tr>
<tr>
<td>ADP</td>
<td>57</td>
<td>42</td>
<td>15</td>
</tr>
<tr>
<td>AMP</td>
<td>18</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>30</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td>ITP</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

a I = MgCl$_2$ (3 mM), NaCl (120 mM), KCl (20 mM), Tris-HCl (40 mM), substrate (3 mM)

b II = same as I plus ouabain (0.5 mM)

c rate of hydrolysis of various substrates expressed as percentages of the rate of hydrolysis of ATP measured under optimum conditions
Table 2. Substrate specificity of ATPase associated with erythrocyte membranes from calves infected with *A. marginale*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Assay medium</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I a (Total)</td>
<td>II b</td>
<td>I minus II</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>100</td>
<td>81</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>53</td>
<td>35</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>11</td>
<td>13</td>
<td>-2</td>
<td></td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>35</td>
<td>33</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>41</td>
<td>20</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

a I = MgCl₂ (3 mM), NaCl (120 mM), KCl (20 mM), Tris-HCl (40 mM), substrate (3 mM)

b II = same as I plus ouabain (0.5 mM)

c rate of hydrolysis of various substrates expressed as percentages of the rate of hydrolysis of ATP measured under optimum conditions
Data presented in Table 3 show the effects of magnesium, sodium, and potassium ions on ATPase activity. In the absence of ions, ATP was not hydrolyzed. Sodium or potassium alone had no effect on ATPase activity, and a combination of these cations alone effected only slight stimulation. A dependency of this enzyme system on magnesium ions was indicated by the marked rise in ATPase activity in the presence of magnesium (3 mM) alone and the lack of activity in its absence. The Mg-dependent ATPase activity of erythrocyte membranes isolated from diseased calves was increased more than three-fold when compared to erythrocyte membranes of normal calves. Assay of ATPase activity with added magnesium increased significantly when sodium (120 mM) and potassium (20 mM) were included in the assay medium, but not when either sodium (140 mM) or potassium (140 mM) were added singly to the assay medium. Maximal ATPase activity was achieved in assay mixtures which contained a combination of sodium, potassium, and magnesium ions. ATPase activity was maximal when the sodium:potassium ions concentration ratio was 6:1 (Table 4). However, the ratio of ATPase activity associated with erythrocyte membranes from normal calves and erythrocyte membranes from diseased calves was lowest at this concentration of sodium and potassium ions. Therefore, increased ATPase activities of erythrocyte membranes from diseased calves under optimal conditions of cation concentration represent minimal increases over normal values since ATPase activity of
Table 3. Effects of magnesium, sodium, and potassium on ATPase activity associated with erythrocyte membranes prepared from normal calves and calves infected with _A. marginale_.

<table>
<thead>
<tr>
<th>Ionic composition of the incubation media (mM)</th>
<th>Total ATPase Activity (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>No ions present</td>
<td>0.0</td>
</tr>
<tr>
<td>(\text{Mg}^{2+}, 3)</td>
<td>116.0</td>
</tr>
<tr>
<td>(\text{K}^+, 140)</td>
<td>0.0</td>
</tr>
<tr>
<td>(\text{Na}^+, 140)</td>
<td>0.0</td>
</tr>
<tr>
<td>(\text{Na}^+, 120 + \text{K}^+, 20)</td>
<td>30.0</td>
</tr>
</tbody>
</table>

\(^a\) nmoles Pi/mg membrane protein/hr
Table 4. Effect of varying sodium and potassium ion concentrations on the magnesium-dependent ATPase activity associated with erythrocyte membranes prepared from normal and *A. marginale*-infected calves.  

<table>
<thead>
<tr>
<th>Na(^+):K(^+) (mM)</th>
<th>ATPase Activity c</th>
<th>I/N d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Infected</td>
</tr>
<tr>
<td>0:0</td>
<td>160</td>
<td>436</td>
</tr>
<tr>
<td>140:0</td>
<td>181</td>
<td>488</td>
</tr>
<tr>
<td>130:10</td>
<td>270</td>
<td>516</td>
</tr>
<tr>
<td>120:20</td>
<td>302</td>
<td>567</td>
</tr>
<tr>
<td>100:40</td>
<td>216</td>
<td>450</td>
</tr>
<tr>
<td>70:70</td>
<td>180</td>
<td>410</td>
</tr>
<tr>
<td>40:100</td>
<td>125</td>
<td>350</td>
</tr>
<tr>
<td>10:130</td>
<td>100</td>
<td>320</td>
</tr>
<tr>
<td>0:140</td>
<td>85</td>
<td>300</td>
</tr>
</tbody>
</table>

\(a\) Mg\(^{2+}\), 3 mM  
\(b\) Constant tonicity (140 mM) in all tubes except 0:0  
\(c\) nmoles Pi/mg membrane protein/hr  
\(d\) ratio of infected to normal
erythrocyte membranes from diseased calves were more refractory to changes in the sodium:potassium ions concentration ratio. The ATPase activity measured at sodium:potassium ions concentration ratio of 120:20 was elevated significantly over either the 140:0 or 0:140 concentrations in both erythrocyte membrane preparations. Thus, this markedly stimulated ATPase activity suggests a Na,K-dependent ATPase activity in addition to the Mg-dependent ATPase activity. However, both components of the total ATPase activity exhibited an absolute requirement for the presence of magnesium ions.

Results on the effect of ouabain on ATPase activity produced with the various ion concentrations are presented in Table 5. Ouabain had no effect on the Mg-ATPase component but inhibited the Na,K-ATPase component. In all cases the ATPase activity of erythrocyte membranes from calves infected with *A. marginale* was approximately two-fold greater than that observed with erythrocyte membranes prepared from normal calves. Inhibition by ouabain of the Mg,Na,K-ATPase activity was 43 per cent with normal erythrocyte membrane preparations and 20 per cent in erythrocyte membranes prepared from diseased calves.

Ouabain inhibition of total ATPase activity is recorded in Table 6. When erythrocyte membrane preparations from diseased calves provided enzyme more than 50 per cent of total ATPase activity was inhibited by 0.1 mM ouabain. Fifty per cent inhibition of total ATPase activity with normal erythrocyte membrane preparations occurred between
Table 5. Effects of magnesium, sodium, and potassium ions on ATPase activity associated with erythrocyte membranes prepared from normal and *A. marginale*-infected calves in the presence and absence of 0.5 mM ouabain.

<table>
<thead>
<tr>
<th>Ionic composition of assay media</th>
<th>ATPase Activity</th>
<th>Normal</th>
<th>Infected</th>
<th>I/N c</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mg</strong>&lt;sup&gt;2+&lt;/sup&gt;</td>
<td></td>
<td>180</td>
<td>452</td>
<td>2.51</td>
</tr>
<tr>
<td><strong>Mg</strong>&lt;sup&gt;2+&lt;/sup&gt; + ouabain</td>
<td></td>
<td>180</td>
<td>460</td>
<td>2.56</td>
</tr>
<tr>
<td><strong>Mg</strong>&lt;sup&gt;2+&lt;/sup&gt; + Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>201</td>
<td>480</td>
<td>2.39</td>
</tr>
<tr>
<td><strong>Mg</strong>&lt;sup&gt;2+&lt;/sup&gt; + Na&lt;sup&gt;+&lt;/sup&gt; + ouabain</td>
<td></td>
<td>137</td>
<td>455</td>
<td>3.32</td>
</tr>
<tr>
<td><strong>Mg</strong>&lt;sup&gt;2+&lt;/sup&gt; + K&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>190</td>
<td>468</td>
<td>2.46</td>
</tr>
<tr>
<td><strong>Mg</strong>&lt;sup&gt;2+&lt;/sup&gt; + K&lt;sup&gt;+&lt;/sup&gt; + ouabain</td>
<td></td>
<td>180</td>
<td>460</td>
<td>2.56</td>
</tr>
<tr>
<td><strong>Mg</strong>&lt;sup&gt;2+&lt;/sup&gt; + K&lt;sup&gt;+&lt;/sup&gt; + Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>302</td>
<td>579</td>
<td>1.92</td>
</tr>
<tr>
<td><strong>Mg</strong>&lt;sup&gt;2+&lt;/sup&gt; + K&lt;sup&gt;+&lt;/sup&gt; + Na&lt;sup&gt;+&lt;/sup&gt; + ouabain</td>
<td></td>
<td>172</td>
<td>465</td>
<td>2.70</td>
</tr>
</tbody>
</table>

a **Mg**<sup>2+</sup>, 3 mM; Na<sup>+</sup>, 120 mM; K<sup>+</sup>, 20 mM

b nmoles Pi/mg membrane protein/hr

c ratio of infected to normal
Table 6. Effect of ouabain on magnesium-dependent and total ATPase activity associated with erythrocyte membranes prepared from normal and \textit{A. marginale}-infected calves.

<table>
<thead>
<tr>
<th>Oubain concentration (mM)</th>
<th>Normal</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg-ATPase $^a$</td>
<td>Total $^b$</td>
</tr>
<tr>
<td>0.0</td>
<td>180</td>
<td>300</td>
</tr>
<tr>
<td>0.1</td>
<td>180</td>
<td>248</td>
</tr>
<tr>
<td>0.2</td>
<td>180</td>
<td>235</td>
</tr>
<tr>
<td>0.5</td>
<td>180</td>
<td>220</td>
</tr>
<tr>
<td>1.0</td>
<td>180</td>
<td>200</td>
</tr>
</tbody>
</table>

$^a$ Mg$^{2+}$, 3 mM

$^b$ Mg$^{2+}$, 3 mM; Na$^+$, 120 mM; K$^+$, 20 mM
0.1 and 0.2 mM ouabain. Moreover, at a ouabain concentration of 1.0 mM, total ATPase activity was decreased to the level of Mg-ATPase with erythrocyte membranes prepared from diseased animals, but total activity of normal erythrocyte membrane preparations was not decreased to a comparable degree.

Although both total ATPase and Mg-ATPase activities of erythrocyte membranes of diseased calves were increased two-fold or more above these activities in normal erythrocyte membranes, the increment due to inclusion of sodium and potassium in the assay medium was the same. That is, the absolute value for the Na,K-ATPase component was identical with both sources of erythrocyte membranes. Since the Mg-ATPase activity was not affected by the addition of ouabain, the decrease in total ATPase at increasing concentrations of ouabain was due to a specific inhibition of the Na,K-dependent ATPase.

Inhibition by calcium ions of total ATPase activity was principally due to its effect on the Na,K-ATPase component and was similar in effect to the inhibition produced by ouabain (Table 7). The Na,K-ATPase was progressively inhibited as the concentration of calcium ions was increased, and was completely abolished by 6 to 10 mM calcium ions. Thus, it appears that calcium ions competed with magnesium ions at the site of Na,K-ATPase activity and were more readily bound, resulting in a selective inhibition of the Na,K-ATPase activity similar to that caused by ouabain. However, as
Table 7. Effect of calcium on magnesium-dependent and total ATPase activity associated with erythrocyte membranes prepared from normal and *A. marginale*-infected calves.

<table>
<thead>
<tr>
<th>Calcium concentration (mM)</th>
<th>Normal Mg-ATPase a</th>
<th>Normal Total b</th>
<th>Infected Mg-ATPase</th>
<th>Infected Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>180</td>
<td>300</td>
<td>480</td>
<td>600</td>
</tr>
<tr>
<td>1</td>
<td>130</td>
<td>220</td>
<td>410</td>
<td>515</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>190</td>
<td>385</td>
<td>490</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>120</td>
<td>380</td>
<td>415</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>100</td>
<td>390</td>
<td>390</td>
</tr>
</tbody>
</table>

a  \( \text{Mg}^{2+}, 3 \text{ mM} \)

b  \( \text{Mg}^{2+}, 3 \text{ mM; Na}^+, 120 \text{ mM; K}^+, 20 \text{ mM} \)
opposed to ouabain, calcium ions did affect the Mg-ATPase activity. At the lower concentrations of calcium ions, the Mg-ATPase activity was inhibited. At concentrations of 2 mM or greater, Mg-ATPase activity did not decrease further as did the total ATPase activity. In erythrocyte membranes isolated from diseased calves there was a slight stimulation of Mg-ATPase activity at increasing concentrations of calcium ions.

Data tabulated in Table 8 show that calcium ions alone could activate ATPase activity but not as effectively as magnesium ions (Table 9). The addition of ouabain had a minimal effect on the ATPase activity measured in the presence of calcium, sodium, and potassium ions, but the higher ATPase activity due to magnesium, sodium, and potassium ions was partially inhibited by ouabain. Maximal stimulation of total and Mg-ATPase activities by magnesium ions occurred at a magnesium ion concentration of approximately 3 mM (Table 9). The ouabain-sensitive Na,K-ATPase component peaked at approximately 1.5 mM magnesium. The ATPase activity peaks observed for the total ATPase and for ouabain-sensitive ATPase activities corresponded to magnesium:ATP concentration ratios of 1:1 and 0.5:1, respectively (Table 9). A greater sensitivity to a deviation from the 1:1 ratio was indicated by the more pronounced decrease of the ouabain-sensitive component relative to the ouabain-insensitive component. These patterns of ATPase stimulation and inhibition were similar with erythrocyte membranes isolated from normal and diseased calves.
Table 8. Activation of ATPase activity associated with erythrocyte membranes prepared from normal and *A. marginale*-infected calves by calcium in the presence and absence of 0.5 mM ouabain.

<table>
<thead>
<tr>
<th>Calcium concentration (mM)</th>
<th>Normal Total</th>
<th>Normal Ouabain-insensitive</th>
<th>Infected Total</th>
<th>Infected Ouabain-insensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>53</td>
<td>50</td>
<td>132</td>
<td>130</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>58</td>
<td>140</td>
<td>135</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>78</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>9</td>
<td>92</td>
<td>90</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>12</td>
<td>90</td>
<td>90</td>
<td>180</td>
<td>180</td>
</tr>
</tbody>
</table>

*a nmoles Pi/mg membrane protein/hr*
Table 9. Effects of various concentrations of magnesium on ATPase activity associated with erythrocyte membranes prepared from normal and *A. marginale*-infected calves in the presence and absence of 0.5 mM ouabain. 

<table>
<thead>
<tr>
<th>Magnesium concentration (mM)</th>
<th>Normal Total</th>
<th>Ouabain-insensitive</th>
<th>Infected Total</th>
<th>Ouabain-insensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>200</td>
<td>110</td>
<td>530</td>
<td>440</td>
</tr>
<tr>
<td>1.0</td>
<td>260</td>
<td>160</td>
<td>560</td>
<td>460</td>
</tr>
<tr>
<td>1.5</td>
<td>305</td>
<td>185</td>
<td>616</td>
<td>492</td>
</tr>
<tr>
<td>2.0</td>
<td>310</td>
<td>200</td>
<td>640</td>
<td>525</td>
</tr>
<tr>
<td>2.5</td>
<td>320</td>
<td>240</td>
<td>670</td>
<td>570</td>
</tr>
<tr>
<td>3.0</td>
<td>330</td>
<td>290</td>
<td>680</td>
<td>600</td>
</tr>
<tr>
<td>6.0</td>
<td>290</td>
<td>270</td>
<td>648</td>
<td>588</td>
</tr>
<tr>
<td>9.0</td>
<td>250</td>
<td>240</td>
<td>620</td>
<td>580</td>
</tr>
</tbody>
</table>

a nmoles Pi/mg membrane protein/hr
Maximal stimulation of total, ouabain-insensitive, and ouabain-sensitive ATPase activities occurred at a calcium ion concentration of 9 mM or a calcium:ATP concentration ratio of 3:1. Moreover, in the presence of calcium ions the amount of hydrolysis of ATP continued to rise as the concentration of calcium ions was increased above that of ATP. However, the enzymic activity due to magnesium ions reached a maximum when the concentration of magnesium ions equalled that of ATP and decreased at higher concentrations. The decrease in ATPase activity at high concentrations of magnesium ions was considerable in the presence of sodium and potassium ions, but was only slight when ouabain was added, particularly in erythrocyte membranes of diseased calves. The ATPase activity associated with erythrocyte membranes of diseased calves was more refractory to changes in ion concentrations or magnesium:ATP ratios.

In addition to studying calcium ions and ouabain, other inhibitors were chosen for comparison of their effects on ATPase of erythrocyte membranes of diseased calves (Table 10). NEM was an effective inhibitor of all ATPase activities at 1.0 mM concentration. This inhibition was preferential for the ouabain-sensitive component. However, at 2.0 mM NEM, the activity of the ouabain-insensitive component was significantly decreased. Although para-chloromercuribenzoate is also a thiol-reactive agent, it was not as effective as NEM as an ATPase inhibitor. At 0.1 mM para-chloromercuribenzoate, ouabain-insensitive activity was preferentially
Table 10. Effect of ATPase inhibitors on ATPase activity associated with erythrocyte membranes prepared from calves infected with *A. marginale*. a

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final conc. (mM)</th>
<th>Total ATPase activity</th>
<th>Ouabain-insensitive ATPase activity</th>
<th>Ouabain-sensitive ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Ethylmaleimide</td>
<td>1.0</td>
<td>84</td>
<td>95</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>62</td>
<td>71</td>
<td>23</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>0.1</td>
<td>98</td>
<td>94</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>89</td>
<td>91</td>
<td>78</td>
</tr>
<tr>
<td>Oligomycin (μg/ml)</td>
<td>0.1</td>
<td>98</td>
<td>99</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>100</td>
<td>99</td>
<td>108</td>
</tr>
</tbody>
</table>

a ATPase activity was nmol Pi liberated/mg membrane protein/hr. Total ATPase activity was 614; total ouabain-insensitive ATPase activity was 505; total ouabain-sensitive ATPase activity was 109.
inhibited; ouabain-sensitive activity was increased. At a higher concentration, all components were inhibited, but to a lesser extent than with NEM. Oligomycin, a respiratory inhibitor, essentially had no effect on ATPase activity of erythrocyte membranes.

In order to compare the kinetics of ATP hydrolysis of erythrocyte membrane preparations, the Michaelis constant for ATP of total ATPase was derived by the Lineweaver-Burk formulation (Figure 7). The line of regression was calculated by the least squares method. The linear relation between the reciprocals of substrate (ATP) concentration and enzyme activity was statistically significant ($r = 0.91$, $p < 0.05$). As estimated from the slope and y intercept, the Km for ATP was $3.87 \times 10^{-4}$ M for erythrocyte membranes prepared from normal calves and $2.47 \times 10^{-4}$ M for membranes prepared from calves with anaplasmosis.

Figure 8 illustrates that total and ouabain-insensitive ATPase activities of erythrocyte membranes from 16 normal and 16 *A. marginale*-infected calves were significantly different ($p < 0.05$) despite the wider variation in activities observed in infected membranes. Data in Figure 9 compares the ratio of Na,K-ATPase activities (ouabain-sensitive) to Mg-ATPase (ouabain-insensitive) activities for these same 32 calves. This ratio was significantly lower in erythrocyte membranes from infected calves due to the increase in ouabain-insensitive ATPase at the peak of the parasitemia.
Fig. 7. Lineweaver-Burk plot for Km of ATP of total ATPase activity associated with erythrocyte membranes prepared from normal (●) calves and calves infected with A. marginale (○).
Fig. 8. ATPase activity (● = total, o = ouabain-insensitive) associated with erythrocyte membranes prepared from 16 normal calves and 16 *A. marginale*-infected calves at peak infection.
Fig. 9. Ratio of sodium-potassium-magnesium dependent ATPase (Na,K-ATPase) activity to magnesium-dependent ATPase (Mg-ATPase) activity associated with erythrocyte membranes prepared from 16 normal (O) calves and 16 calves infected with A. marginale (●) at peak infection.
These results led to an investigation of the changes in the various components of ATPase activity through the course of the infection in three calves (Fig. 10, 11, 12). After pre-infection values were obtained, the disease was followed and divided into clinically recognizable stages. Total ATPase activity was observed to increase significantly early in the infection after the initial appearance of Anaplasma bodies (Fig. 10). During the middle of the infection when *A. marginale* counts were increasing slowly, total ATPase activity consistently decreased, but remained above pre-infection values. Thereafter, total ATPase activity increased rapidly as *A. marginale* counts increased, and reached its maximum at the peak of the infection. The increase of ouabain-insensitive ATPase activity correlates with that of total ATPase activity (Fig. 11). However, ouabain sensitivity decreased from 42 per cent to 19 per cent during the disease. Data presented in Figure 12 shows that the ouabain-sensitive component did not deviate significantly from pre-infection values during the course of the infection.

No significant change in ATPase activity or per cent inhibition by ouabain was detected in normal asplenic calves utilized as controls over time periods comparable in length to those of the course of infection with anaplasmosis.

In order to rule out the possibility of changes in ATPase activity due primarily to the anemia produced in anaplasmosis, experimental anemia was induced in two calves
Fig. 10. Total ATPase activity associated with erythrocyte membranes prepared from three calves before and during the course of infection with A. marginale. (N = number of determinations, $\bar{x} = \pm 1$ S.D.)
Fig. 11. Ouabain-insensitive ATPase activity associated with erythrocyte membranes prepared from three calves before and during the course of infection with A. marginale. (N = number of determinations, \( \bar{x} = \pm 1 \) S.D.)
Ouabain-sensitive ATPase activity associated with erythrocyte membranes prepared from three calves before and during the course of infection with *A. marginale*. (N = number of determinations, $\bar{x} = \text{mean} \pm 1 \text{ S.D.}$)
by phlebotomy and injection of phenylhydrazine. As presented in Table 11, total ATPase activities during these two anemic conditions did not deviate significantly from normal values although the anemia was comparable in severity to that produced by *A. marginale* infection. In both cases the anemia was produced in a relatively short time period to prevent the complications of erythropoietic response and the resulting increase in numbers of immature erythrocytes.

These results indicated that a portion of the increased ATPase activity in infected erythrocytes might be associated with *A. marginale*. Therefore, an immunological system was employed to determine whether the enzymatic activity being measured could be attributed to *A. marginale* rather than solely to the erythrocyte membranes (Table 12). A scheme was utilized to quantitate the per cent decrease in total ATPase activity after incubation of membrane preparations with various antiserums. Rabbit antiserum prepared against pooled samples of normal erythrocyte membranes (anti-N) reduced ATPase activity in erythrocyte membranes from normal calves by 56.8 per cent but only decreased ATPase activity in erythrocyte membranes prepared from *A. marginale*-infected calves by 16.1 per cent. Conversely, rabbit antiserum prepared against pooled samples of erythrocyte membranes prepared from diseased calves (anti-I) caused a 40.3 per cent decrease in ATPase activity when incubated with membranes prepared from diseased calves and a 21.6 per cent
Table 11. Effect of experimental anemia induced by phenylhydrazine injections and phlebotomy on total ATPase activity associated with erythrocyte membranes prepared from normal calves.

<table>
<thead>
<tr>
<th>Day</th>
<th>Phenylhydrazine Injections</th>
<th>Phlebotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCV  a</td>
<td>Activity b</td>
</tr>
<tr>
<td>0</td>
<td>32</td>
<td>254</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>271</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>242</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>279</td>
</tr>
<tr>
<td>4</td>
<td>Died</td>
<td>ND</td>
</tr>
</tbody>
</table>

a  Packed cellular volume
b  nmoles Pi/mg membrane protein/hr
Table 12. Changes in total ATPase activity resulting from the incubation of erythrocyte membranes prepared from normal and *A. marginale*-infected calves with various rabbit antiserums.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total ATPase activity a</th>
<th>Absolute decrease</th>
<th>Per cent decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>370</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>+ NRS b</td>
<td>350</td>
<td>20</td>
<td>5.4</td>
</tr>
<tr>
<td>+ AN c</td>
<td>160</td>
<td>210</td>
<td>56.8</td>
</tr>
<tr>
<td>+ AI d</td>
<td>290</td>
<td>80</td>
<td>21.6</td>
</tr>
<tr>
<td>Infected</td>
<td>620</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>+ NRS</td>
<td>560</td>
<td>60</td>
<td>9.7</td>
</tr>
<tr>
<td>+ AN</td>
<td>520</td>
<td>100</td>
<td>16.1</td>
</tr>
<tr>
<td>+ AI</td>
<td>370</td>
<td>250</td>
<td>40.3</td>
</tr>
</tbody>
</table>

a nmoles Pi liberated/mg membrane protein/hr
b normal rabbit serum
c rabbit antiserum prepared against normal calf erythrocyte membranes
d rabbit antiserum prepared against erythrocyte membranes prepared from calves infected with *A. marginale*
decrease with erythrocyte membranes prepared from normal calves. Normal rabbit serum (NRS) was not found to possess ATPase activity and did not decrease ATPase activity to the same degree as immune serum.

Another attempt to correlate ATPase activity with *A. marginale* followed differential and density gradient centrifugation of a marginal body preparation (Table 13). The initial aliquot applied to the density gradient was assayed for ATPase activity in the presence and absence of ouabain and these values taken as 100 per cent. By this method a mean of 28.3 per cent of total ATPase activity was found associated with partially-purified *A. marginale*; whereas, 41.4 per cent was associated with the faster sedimenting membrane fraction. A total recovery of ATPase activity was not effected due to the dilution effect inherent in this procedure. Recovered ouabain-insensitive ATPase activity was evenly distributed between the two fractions. However, 78.9 per cent of ouabain-sensitive activity was recovered in the membrane fraction and only 7.6 per cent was recovered in the marginal body fraction.
Table 13. ATPase activity of preparations of partially-purified *A. marginale* and erythrocyte components obtained by sucrose density gradient centrifugation.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total ATPase activity a</th>
<th>Ouabain-insensitive ATPase activity</th>
<th>Ouabain-sensitive ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial b</td>
<td>935</td>
<td>750</td>
<td>185</td>
</tr>
<tr>
<td><em>A. marginale</em></td>
<td>264</td>
<td>250</td>
<td>14</td>
</tr>
<tr>
<td>Membranes</td>
<td>387</td>
<td>241</td>
<td>146</td>
</tr>
</tbody>
</table>

a  nmoles Pi liberated/sample

b  suspension of *A. marginale* prior to sucrose density gradient centrifugation
DISCUSSION

The results presented in this dissertation confirm that a magnesium-dependent, ouabain-insensitive ATPase and a sodium-potassium-dependent, ouabain-sensitive ATPase is associated with the bovine erythrocyte membrane (77). Similarly, characteristics and conditions for optimal activity and inhibition of the enzyme system were comparable to those described for the membrane ATPase of other mammalian erythrocytes (5, 65, 66, 105, 134). The results presented here confirm that the bovine erythrocyte, like that of other species, possesses an enzyme system with the characteristics of those ATPases required for active transport and for metabolism (12, 44, 93, 153, 161).

Since no ATPase activity was detected in blood plasma or cytoplasmic constituents, the conclusion can be made that the enzyme system is an integral component of the erythrocyte membrane. The methods employed to prepare erythrocyte membranes for assay of ATPase did not solubilize membrane bound enzymes or adversely affect enzymatic activity. Although the electrochemical method of assaying ATPase activity was not satisfactory for all parameters in this study, it was satisfactory to confirm a three-fold increase in the initial rate of ATP hydrolysis by ATPase of erythrocyte membranes from diseased calves over that obtained by ATPase of erythrocyte membranes from normal calves.
A chemical method for measuring ATPase activity was employed to determine the effects of *A. marginale* infection upon the various components of the enzyme system.

The pH and temperature required for maximum ATPase activity were similar to values previously reported (67, 77, 82) and did not change during infection.

There is wide disagreement in the literature on the question of substrate specificity of ATPases (153). Some of the discrepancies could be due to differences between tissues. Moreover, it is difficult to compare results in detail because of the variety of experimental conditions employed; especially in membrane preparation procedures, reagent concentrations, and methods of assay of ATP hydrolysis. Such comparisons are further complicated by the lack of information in the literature for the normal bovine erythrocyte.

Under conditions which were optimum for ATP hydrolysis in this system, the hydrolysis of ADP was less (approximately half) of that of ATP, and might have been caused by an adenylate kinase. However, the extent of inhibition of ADP hydrolysis by ouabain was greater than that of ATP in both normal and infected membranes. As the amount of ATP decreases in the assay medium, due to hydrolysis by ATPase, ADP may serve as a suitable substrate for the enzyme. Nevertheless, in this study, less than one per cent of the terminal phosphate of ATP was hydrolyzed during a typical assay. Therefore, it appears that an appreciable amount of
ADP hydrolysis was attributable to the Na,K-ATPase component of the ATPase enzyme system. The specificity of the ATPase system for ADP increased during the infection although the absolute amount of Na,K-ATPase did not increase. The hydrolysis of ITP by normal erythrocyte membranes was insignificant. However, ITP hydrolysis increased appreciably in erythrocyte membranes prepared from diseased calves but inhibition by ouabain remained constant. This indicated an increase in specificity for ITP as a substrate for both the Mg- and Na,K-ATPases in the erythrocyte membranes from calves infected with *A. marginale*. AMP and pyrophosphate were hydrolyzed very slowly by ATPase associated with membranes prepared from normal and diseased calves. Apparently anaplasmosis has little or no effect on hydrolysis of these substrates by ATPase.

The cation studies showed that ATPase activity has an absolute requirement for magnesium ions. However, maximal ATPase activity requires magnesium, sodium, and potassium ions. Thus, the ATPase system of normal erythrocyte membranes and erythrocyte membranes from diseased calves was shown to be composed of at least two active components. This is in agreement with investigations employing other membrane systems (5, 63, 105). The activity of the Na,K-ATPase component was responsive to variations in the relative concentrations of sodium and potassium ions. An optimal sodium:potassium concentration ratio is consistent with the demonstration of specific binding sites for each
cation in human erythrocyte membranes (153). Deviation from the optimal sodium:potassium ratio of 6:1 resulted in decreased ATPase activity which was probably due to competition of each cation for the alternate binding site. Assymetric stimulation of ATPase by these cations was assumed in these studies although these experiments did not permit variation of cation concentration on each side of the membrane. However, ATPase activity in erythrocyte membranes from calves with anaplasmosis was not as responsive to variations in cation concentrations and ratios, a finding which could have been effected by a change in specificity of the sodium and potassium binding sites.

The effects of ouabain on ATPase activity were similar to those previously reported for the bovine erythrocyte membrane (77). Hence, ouabain was observed to inhibit the Na,K-ATPase but not the Mg-ATPase. Inhibition was maximal at a concentration of approximately 1.0 mM ouabain in erythrocyte membranes from diseased animals and at higher concentrations in normal erythrocyte membranes.

Experimental results showed that calcium ions could replace magnesium ions to activate the ouabain-insensitive ATPase activity although not as effectively. Calcium ions could not replace magnesium ions for activation of the ouabain-sensitive ATPase component. This supports the conclusion that Na,K-ATPase has an absolute requirement for magnesium ions for maximal activity. Moreover, increasing concentrations of calcium ions preferentially inhibited
the Na,K-ATPase activity, and no synergistic effect was observed with combinations of calcium and magnesium ions. Therefore, it appears that bovine erythrocyte membranes do not possess any significant Ca-ATPase activity. Similar results were obtained with membranes prepared from normal and diseased calves.

Maximal activity was observed with a magnesium:ATP concentration ratio of 1:1. However, the two components of ATPase varied in their response to a change in this ratio. As magnesium ions were increased above the 1:1 ratio with respect to ATP, ATPase activity of both components were decreased, especially Na,K-ATPase. Therefore, magnesium ions in the free state rather than the Mg-ATP complex were inhibitory to ATPase. However, ATPase activity of erythrocyte membranes from diseased calves was affected to a lesser extent by free magnesium ions.

The effects of various inhibitors on ATPase activity was not conclusive. Two thiol-reactive reagents exhibited variable and opposite effects on the component activities of ATPase. A respiratory inhibitor, oligomycin, had no effect. No difference was detected between normal erythrocyte membranes and erythrocyte membranes from diseased calves with any of the inhibitors.

The Km for ATP of $3.87 \times 10^{-4}$ M for normal calf enzyme differed significantly from the $2.1 \times 10^{-4}$ M value previously reported for the adult bovine erythrocyte ATPase (77). The Km for ATP for erythrocyte membranes from
diseased calves decreased to $2.47 \times 10^{-4}$ M which reflects the increased activity of the ATPase enzyme system at the peak of the infection. An increased affinity for the substrate ATP could have been due to ATPase synthesized by *A. marginale* or a change in the ATPase of the normal erythrocyte membrane.

Subsequent studies showed that ATPase activity increased significantly during the early stage of the infection immediately after the initial detection of *A. marginale* in the erythrocytes. Since the per cent of infected erythrocytes at this stage was less than 5 per cent, the total volume of *A. marginale* with respect to membranes would be insignificant. Therefore, increased ATPase activity would appear to be due to a change in the ATPase of the erythrocyte membrane. The life cycle of *A. marginale* is not known, but after the initial appearance of the organism in the peripheral circulation, activity of the organism is greatly increased. This increased activity includes multiplication of the organism and its appearance in additional erythrocytes. The action of the organism directed towards the erythrocyte membrane may cause changes in the structural integrity of the membrane and lead to derangements in the normal cation gradients across the membrane. This is reflected in the increase in cation permeability and ATPase activity observed at this stage of the infection (57, 58). However, as in all pathological conditions, any observed change is usually a manifestation of a complex array of
interrelated forces. At this stage of infection, various A. marginale-specific antibodies, as well as erythrocyte-bound and free-serum autoantibodies and opsonins, can be detected (15, 23, 84, 92, 126, 127). These humoral immune responses are involved in the development of a progressive autohemolytic anemia in anaplasmosis (15, 84, 126). It has been shown that immune injury to the erythrocyte membrane can cause an activation of red cell membrane ATPase with a resulting significant increase in its activity (102). Such an alteration of the membrane at its external surface may unmask the enzyme system and increase its availability to the substrate or increase cation permeability of the membrane. Furthermore, more direct conformational change (structural modifications) in both the membrane and the protein of the enzyme would possibly lead to a change in its specificities and optimal conditions for activation and inhibition. Damage of the red cell membrane by various physical and chemical treatments of membranes effect these results (10, 22, 64, 90, 137, 157). ATPase activity is commonly used as a measure of change in membrane structure and integrity during preparation of erythrocyte membranes (10, 64, 137). Increased ATPase activity has been associated with other pathological autoimmune hemolytic anemias such as hereditary spherocytosis (70, 94, 97, 98) and paroxysmal nocturnal haemoglobinuria (8). ATPase activity does not deviate from normal in primary refractory anemias (101). No change in ATPase activity was detected in this investigation when anemia was experimentally induced. Therefore,
the changes in ATPase activity reported here may be a direct consequence of anaplasmosis rather than the secondary anemia induced during the hemolytic crisis.

No plausible explanation can be given for the decrease in ATPase activity during the middle stage of anaplasmosis. However, the mean ATPase activity was increased significantly and progressively as the per cent of parasitized erythrocytes increased. Maximal ATPase activity was measured at the peak of the infection. No values for ATPase activity are given for the convalescent period since in all cases the calves died during the hemolytic crisis.

Although total ATPase activity was increased more than two-fold, the Na,K-ATPase activity did not deviate significantly from normal either at the peak or during the infection. However, as noted previously, some of its characteristics were changed. The increased activity was produced solely by the three-fold increased activity in the Mg-ATPase. A similar increase in Mg-ATPase occurs in some cases of hereditary spherocytosis (89). At the peak of the infection with A. marginale, more than 56 per cent of the erythrocytes contained the organism. Although the volume of A. marginale compared to that of the erythrocyte would be low, active proliferation and increased metabolic activity of the parasite could have profound effects upon the structure and function of the host cell.

It has been reported that A. marginale infection causes a decrease in phospholipid concentration of
erythrocytic stroma (122), increases in sialic acid concentration, and electrophoretic mobility of parasitized erythrocytes (37), changes in fatty acid composition of parasitized erythrocytes (36), and increased cation permeability of the erythrocyte (57). These investigations, as well as others (33, 34), have shown that bovine erythrocytes parasitized by *A. marginale* undergo a change in ultrastructure of the erythrocytic membrane (33, 34). Moreover, *A. marginale* infection produces a progressive decrease in acetylcholinesterase activity in parasitized erythrocytes which was correlated to the resulting anemia and osmotic fragility (145).

In hereditary spherocytosis it has been suggested that the increased ATPase activity and morphologic and physiologic changes of erythrocytes is secondary to a dynamic structural defect dependent on ATP and cation balance of the cells (81, 88, 147). The major role of ATP in maintenance of erythrocyte viability relates to its preservation of the shape of the erythrocyte (147). A depletion of membrane phospholipids in hereditary spherocytosis was correlated to the excessive cation flux (69). It was suggested that the membrane structural integrity was altered due to the high turnover rate of the cation molecules through the membrane, and consequently leads to hemolysis of the cells. Sodium influx was shown to increase 35 percent with no increase in intracellular sodium because of the compensatory increase in ATPase activity.
Malarial infection has been shown to increase membrane permeability in both parasitized and nonparasitized cells which results in an increased susceptibility of the cells to osmotic lysis (41). However, an increase in intracellular sodium in all cells occurs at levels of parasitemia less than 10 per cent, which is primarily due to the impaired active transport mechanisms and secondarily a consequence of the 50 per cent increase in sodium influx. Moreover, an increase in ATP and a decrease in ATPase has been measured in certain malarial infections (50, 128).

Although ATP concentration during A. marginale infection has not been determined, the concentrations of all cations are not altered during the infection (57), but cation permeability of the cells is increased. Therefore, as opposed to malarial infections, there is a compensatory increase in ATPase activity in anaplasmosis which prevents an abnormal accumulation of sodium within the cells. This effect is similar to that seen in hereditary spherocytosis, and one would assume that this would lead to a depletion of intracellular ATP.

A decrease in ATP, coupled with the aforementioned ultrastructural changes in the erythrocyte membrane, would be a contributing factor to the hemolytic anemia observed in clinical anaplasmosis.

ATPase activity of A. marginale has been reported (58). It has been suggested that A. marginale exerts a definite influence upon the metabolism of the erythrocyte membrane
and that this influence is manifested in the energy dependent system of the cell. The manner by which the organism exerts its effect upon metabolism and energy production of the erythrocyte is open to conjecture.

In the present study, significant ATPase activity was found in preparations of partially-purified \textit{A. marginale}. The distribution between the two components of ATPase activity was the same as that measured in membranes harvested at the peak of \textit{A. marginale} infection. After density gradient centrifugation, significant ATPase activity was found in fractions enriched with \textit{A. marginale}. However, this fraction had a significantly reduced amount of ouabain-sensitive ATPase activity. Most of the ouabain-sensitive ATPase activity was recovered in fractions which contained erythrocyte membranes. Although the fractions which contained \textit{A. marginale} were not devoid of erythrocyte membrane material, decreased sensitivity of ATPase to ouabain in this fraction is significant. Sonication was included in the method employed to partially-purify \textit{A. marginale}. Short term sonication has been reported to preferentially decrease Na,K-ATPase and increase total ATPase which results in an increase of the ouabain-insensitive ATPase to ouabain-sensitive ATPase ratio (149). However, partially-purified \textit{A. marginale} possessed the same level of ouabain-sensitive ATPase activity as erythrocyte membranes prepared by hypotonic hemolysis. It is possible that those membranes most closely associated with \textit{A. marginale} may have changed more
drastically with respect to their content of the two components of ATPase activity.

Additionally, an immunological technique related ATPase activity to *A. marginale*. Antiserum prepared against normal erythrocyte membranes reduced ATPase activity in normal erythrocyte membranes to a greater extent than similar treatment of erythrocyte membranes isolated from calves with anaplasmosis. An opposite effect was measured after treatment with antiserum prepared against erythrocyte membranes from diseased calves. These data suggest that at least part of the increase in ATPase activity during anaplasmosis is contributed by *A. marginale*. Altered antigenic specificity may be explained by alteration of antigenic determinants of the erythrocyte membrane during the infection. Changes in membrane composition and the presence of erythrocyte-bound autoantibodies as a result of the autoimmune humoral response could be responsible for altered antigenic specificity. Therefore, this system would not lead to the conclusion that the increased ATPase activity can be attributed to the *Anaplasma* organism.
SUMMARY AND CONCLUSIONS

A study was initiated to determine the effects of anaplasmosis on the ATPase activity of the erythrocyte membrane. In addition, partially-purified marginal bodies were assayed to determine their contribution to the substantial increase in total ATPase activity observed in the infection.

Significant changes were observed in the characteristics of ATPase activity both at the initial manifestation and peak of *A. marginale* infection. These changes were discussed in relation to the previously reported immunologic, physiologic, pathologic, and metabolic responses observed in the disease which contribute to ultrastructural alteration of the erythrocyte membranes.

The effects of increases in ATPase activity was related to an excessive requirement for ATP for active transport as a consequence of modification of membrane properties. Reduction of intracellular levels of ATP would result in a decrease in red cell viability and contribute to the acuteness of the hemolytic anemia syndrome observed in anaplasmosis.
LITERATURE CITED


VITA

Hollis Utah Cox was born in Holdenville, Oklahoma, on March 4, 1944. He was educated in public school systems and graduated from high school in Jacksonville, Arkansas. In September, 1961, he entered Oklahoma State University of Agriculture and Applied Sciences and earned a Bachelor of Science degree in Agriculture in May, 1965, and a Doctor of Veterinary Medicine degree in May, 1967.

After being discharged from the United States Air Force, he was appointed Instructor in the Department of Veterinary Science, Agricultural Experiment Station, Louisiana State University, in February, 1970. In January, 1972, he received a Postdoctoral Research Fellowship through the National Defense Education Act.

He is married to the former Terry Lovelace of Plaquemine, Louisiana, and is presently a candidate for the Doctor of Philosophy degree in Microbiology with a minor in Biochemistry.
Candidate: Hollis Utah Cox

Major Field: Microbiology

Title of Thesis: ATPase Activity Associated with Bovine Erythrocyte Membranes in Anaplasmosis

Approved:

[Signatures]

Major Professor and Chairman

James B. Brayner

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

EXAMINATION AND THESIS REPORT

Date of Examination:

July 19, 1973