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The Ohio State University, Ph.D., 1972
Microbiology

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ERYTHROCYTE MEMBRANE ALTERATIONS AND ASSOCIATED PLASMA CHANGES INDUCED BY PLASMODIUM GALLINACEUM INFECTION

DISSERATION

Presented in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy in the Graduate School of the Ohio State University

By

Thomas M. Seed, B.A., M.Sc.

* * * * *

The Ohio State University
1972

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>PUBLICATIONS</td>
<td>iii</td>
</tr>
<tr>
<td>FIELDS OF STUDY</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
</tbody>
</table>

**Chapter**

<table>
<thead>
<tr>
<th>Chapter</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td>15</td>
</tr>
<tr>
<td>III. RESULTS</td>
<td>32</td>
</tr>
<tr>
<td>IV. DISCUSSION</td>
<td>100</td>
</tr>
<tr>
<td>V. SUMMARY</td>
<td>117</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>119</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>120</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

**Table** | **Page**
---|---
1. Osmotic resistance of red cells obtained from chickens prior to and during the course of *Plasmodium gallinaceum* infection | 55
2. Mean red cell volume in chickens with *P. gallinaceum* infection | 65
3. The relationship of parasitization to red cell size and volume | 68
4. Sodium and potassium concentrations determined by atomic absorption spectrophotometry | 71
5. Sodium and potassium concentrations determined by atomic absorption spectrophotometry of erythrocytes of chickens injected with phenylhydrazine | 74
6. Red cell ATPase activity in erythrocytes of chickens with *Plasmodium gallinaceum* infection | 79
7. The relationship of hemolytic crisis and recovery to ATPase activity in erythrocytes | 81
8. Phospholipid and cholesterol composition of erythrocytes of normal and *P. gallinaceum* infected chickens | 84
9. The effect upon red cell phospholipid and cholesterol concentrations of injecting chickens with phenylhydrazine | 87
10. Changes in plasma chemistry of chickens infected with *P. gallinaceum* | 90
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Electron micrographs of extracellular and intracellular forms of <em>Plasmodium gallinaceum</em></td>
<td>3</td>
</tr>
<tr>
<td>2. Photomicrographs of red blood cells obtained from chickens prior to infection and during the prepatent, middle patent, late patent, and recovery phases of <em>Plasmodium gallinaceum</em> infection</td>
<td>34</td>
</tr>
<tr>
<td>3. Carbon replicas of erythrocytes from healthy and <em>P. gallinaceum</em> infected chickens</td>
<td>39</td>
</tr>
<tr>
<td>4. Electron micrographs of carbon replicas of erythrocytes from <em>P. gallinaceum</em> infected chickens showing the relationship of parasitization to erythrocyte pathology</td>
<td>42</td>
</tr>
<tr>
<td>5. The morphology of carbon replicated red cells of a chicken recovering from malaria and in blood of a chicken suffering from drug induced, non-infectious anemia</td>
<td>45</td>
</tr>
<tr>
<td>6. Scanning electron micrographs of erythrocytes from a healthy chicken and from <em>P. gallinaceum</em> infected chickens</td>
<td>48</td>
</tr>
<tr>
<td>7. Partial cross sectional views of freeze-cleaved normal and infected erythrocytes</td>
<td>50</td>
</tr>
<tr>
<td>8. Electron micrographs of thin-sectioned normal and infected erythrocytes</td>
<td>52</td>
</tr>
<tr>
<td>9. Photomicrographs of normal and infected erythrocytes suspended in hypotonic salt solution</td>
<td>58</td>
</tr>
<tr>
<td>10. The osmotic fragility of red cells from animals stressed by extensive bleeding or by injection of phenylhydrazine</td>
<td>61</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>11. Red cell volume distributions, determined by electronic particle counting and sizing device, of erythrocytes in samples from normal and infected chickens</td>
<td>63</td>
</tr>
<tr>
<td>12. Cytochemical localization of ATPase activity in normal and infected erythrocytes</td>
<td>76</td>
</tr>
<tr>
<td>13. An osmogram showing the fragility of normal chicken erythrocytes treated with plasma from chickens in the middle and late patent phase of <em>P. gallinaceum</em> infection</td>
<td>93</td>
</tr>
<tr>
<td>14. An osmogram showing the fragility of normal chicken erythrocytes treated with fractions of normal and late patent phase plasma</td>
<td>96</td>
</tr>
<tr>
<td>15. Effect of incubation in whole plasma and plasma ultrafiltrates on survival of Cr51 labeled erythrocytes</td>
<td>99</td>
</tr>
</tbody>
</table>
INTRODUCTION

Malaria is a disease caused by obligately intracellular protozoa. Parasitologists place the protozoa which cause malaria in the subphylum Sporozoa within the genus *Plasmodium*. Species of this genus have as primary hosts man and other primates, rodents, birds and reptiles. It is in the primary host that parasitization causes disease. The disease is characterized by hemolytic anemia and fever. The secondary host is a blood sucking, dipterous insect. The secondary host acts as a vector, transmitting the microorganisms from one vertebrate host to another. In the primary and secondary hosts a cyclic developmental process occurs. During this process the morphology and physical-chemical properties of the organisms vary. The mode of reproduction within each of the hosts is distinct. The parasite multiplies within the vertebrate host's cells by an asexual process called schizogony. The schizogonic cycle begins when infective extracellular parasites, called merozoites (Fig. 1A, EP) penetrate the host erythrocytes and develop intracellularly (Fig. 1B, IF). The parasites utilize hemoglobin as a nutrient source. The intracellular parasites grow and divide, producing
Figure 1. Electron micrographs showing: (A) the morphology of the extracellular *Plasmodium gallinaceum* parasites (EP) by the carbon replica technique and (B) the surface morphology of intraerythrocytic plasmodia (IP) by the freeze-cleave method. Abbreviations: EP, extracellular *P. gallinaceum* parasites; RBC, red blood cell; cp, cytoplasmic membrane of red cell; hb, hemoglobin; IP, intraerythrocytic plasmodia; HN, host cell nucleus. Line markers equal 10 µm for Figure 1A and 1 µm for Figure 1B.
new crops of young infectious merozoites which are released into the circulation upon lysis of the host red cells. The result of parasite growth in the vertebrate host is red cell destruction and production of anemia. Within the insect the parasite reproduces by a sexual process in which gametocytes produced originally in the blood of the primary host mature and fuse to produce a zygote. Through a process called sporogony the zygotes undergo reduction division and produce thousands of uninucleate sporozoites. It is these spindle shaped sporozoites which the insect transmits to the primary host while biting and sucking blood.

Malaria in man is caused by any one of four species of plasmodia, i.e. Plasmodium vivax, P. ovale, P. malariae and P. falciparum. All four species have as vectors mosquitoes of the genus, Anopheles. The four species of Plasmodium are defined on the basis of the morphology of the intracellular parasites as seen by light microscopy of stained blood films and tissue sections and by the symptoms displayed by the infected host. Plasmodium vivax is the most widely spread of the malarial parasites which infect man. It is found in both tropical and temperate areas. "Vivax" malaria is often called "benign tertian" malaria because the febrile signs recur characteristically at 48 hour intervals and because the infection is relatively mild. In contrast, Plasmodium falciparum the other common plasmodium which induces
the "malignant tertian" type of malaria, is extremely virulent. The disease in untreated cases runs a short course usually resulting in death. The remaining two species, *P. malariae* and *P. ovale* are of less importance in human medicine than *P. vivax* and *P. falciparum*, because of their low incidence and the lack of severity of the infections. As does the "vivax" malaria parasite both, *P. ovale* and *P. malariae* produce chronic, debilitating diseases characterized by recurrent fevers. The duration between fevers is 48 hours in "ovale" malaria and 72 hours in "malariae" infections.

Malaria as a disease is unimportant in veterinary medicine because the disease does not occur in any important domestic animal except the chicken and there only rarely. However malarial parasites which infect rats, birds and primates are extremely important in experimental systems in which these parasites serve as models for the study of human malaria. These experimental models are extremely important to malariologists since the use of man in experimentation is not always practical. Since rodents and birds are inexpensive, readily available and easy to handle, many workers prefer to use the latter animals instead of monkeys or apes. Until the discovery of the rodent plasmodia in 1948 much of the antimalarial drug screening carried out in the U.S. was with the avian malarial parasites. Still today many studies
of the biology of plasmodia are done on the avian and rodent malarias. *Plasmodium gallinaceum* infection in domestic chickens, the experimental host-parasite model system utilized in this work, has served in the past and still serves today as a reasonable model system for study of malaria in man. Similarities between the avian parasites' developmental stages, basic physiology, insect vectors, antigenic relationships and general pathology, and those of the human parasite justify its use. In addition, the system is a good one for fundamental studies on the nature of obligate intracellular parasitism.

Prior to the institution of the international malaria eradication program several decades ago, malaria was probably the most serious health problem of the peoples of the world. In 1946 three million deaths were attributed to malaria. This number decreased to about one million by 1958 as a result of a cooperative, international program (Chandler and Reed, 1961). During the late 1950's medical health officials had high hopes of quickly conquering the disease with a limited expenditure of funds and effort. Such optimism was short lived since it was soon realized that the anti-malarial measures used so successfully in Europe and the United States were ineffective in many areas of the tropics and subtropics (Garnham, 1967). For example, Ceylon which made enormous strides in malaria
eradication in the 50's is now reporting over one million cases of malaria per year (Fernandez-Maruto, 1970). In isolated areas such as the Indian state of Assam where the incidence rate was on the decline in the previous decade the case rate has increased from 12.5:10,000 to 31.5:10,000 (Gilroy, 1970).

As a result of involvement in the Vietnam War the peoples of the United States have again recognized malaria as a serious health problem. In 1968, 12,000 cases of malaria occurred in personnel in the armed forces. These infections caused a loss of one quarter million man-working days (Gochenour, 1969). The United States Public Health Service reported that 3981 cases of malaria occurred in the U.S.A. during 1970 (C.D.C., Morbidity, Mortality Report; 1971). This figure is the highest since 1959.

Eradication of malaria will depend on continued development of effective new insecticides and chemotherapeutic agents to eliminate drug resistant forms of vectors and parasites as they arise. The elimination of malaria as a major world health problem will not be as simple as was thought a decade ago. However if appropriate controls are applied consistently in those regions where eradication control measures have already been implemented and control measures are initiated in other malarious areas then perhaps the goal of malaria
eradication can be reached.

A major component of the pathology of plasmodial infection is damage to the host's red cells. This damage is a consequence of the plasmodial infection regardless of whether it is a direct result of parasitic invasion or an indirect effect resulting from an altered host response. Drug therapy has proven most useful in halting parasite growth and multiplication, however such treatment fails to correct already established tissue pathology. Improved methods of treatment of malarial infections will most surely combine drug therapy aimed at parasite destruction with treatment to block tissue alteration and to restore the function of damaged tissue to normal. To develop such treatments, researchers will have to be supplied with basic data concerning the types and mechanisms of red cell pathology which occur in individuals with malaria. The changes in the limiting plasma membrane of the infected erythrocytes are particularly important in the pathology of malaria. It is because this type of pathology is important that a descriptive study of it was made a part of the subject matter of this thesis.

Attempts to understand the mechanisms of erythrocyte destruction in malaria have yielded libraries of literature but just one accepted concept. That is that parasitization of erythrocytes by plasmodia results in pathology which leads to erythrocyte destruction. A
number of investigators have stated in their publications that the major portion of red cell destruction in malaria is the direct result of parasitization (Kitchen, 1949; Kreier, 1969). To what extent non-parasitized red cells are altered and sequestered at an increased rate has been debated for many years. Taliaferro in 1936 reported that during the acute phase of experimental primate malaria large numbers of non-parasitized red cells were phagocytized by splenic macrophages. Similar findings were reported in studies of avian (Taliaferro and Taliaferro, 1955) and rodent malarias (Zuckerman, 1966). Reports that red cell destruction is in excess of that possible with the number of parasites in the blood have stimulated the formulation of a number of hypotheses which attempt to explain how noninfected erythrocytes are destroyed during malaria. The first hypothesis formulated was based on the premise that toxic products circulate within diseased individuals and alter noninfected erythrocytes in such a way as to shorten their survival. Cell altering substances suggested were (1) metabolic byproducts of developing intracellular parasites, such as malaria pigment which is the unmetabolized heme portion of hemoglobin. Hemozoin has been reported to be a hemolytic factor which is potentiated in the presence of lipodal substance derived from degenerating diseased tissues (Laser, 1948, 1950); (2) plasmodial aggressins
(Zuckerman, 1964; Riley and Kaigraith, 1961; Ladda et al., 1969) similar to host cell penetrating factors isolated from *Toxoplasma* parasites (Lycke et al., 1969); (3) inactive enzymes within the host's circulatory system such as trypsinogen, which are activated with onset of tissue pathology (Kreier, 1969).

The second hypothesis which attempted to explain the mechanism of "excess erythrocyte destruction" was an outgrowth and extension of the first hypothesis. Simply stated, it was proposed that red cell surfaces are altered antigenically by either absorption of circulating parasite products (Zuckerman, 1964) or by enzymatic action (Zuckerman, 1964; Kreier et al. 1966; Seed and Kreier, 1969) causing the host's immunological system to respond to these tissues as foreign. The concept of autoimmunization and antibody mediated destruction of nonparasitized cells during infection was popular during the last decade but never conclusively demonstrated by experimentation. Much of the evidence for proposing autoimmunity as a mechanism of red cell destruction was circumstantial, such as finding autoantibodies to trypsinized erythrocytes within sera of malarious experimental animals (Kreier et al. 1966; Barrett et al. 1970) and the localization of globulins bound to erythrocytes during the acute phase of malaria (Zuckerman, 1960; Zuckerman and Spira, 1961; Gautam et al. 1970). To date, the nature of the role of autoantibodies in the
pathogenesis of malaria has not been clarified although it is almost certainly not the destruction of undamaged erythrocytes (George et al., 1966; Kreier and Leste, 1968; Kreier, 1969). A third hypothesis was formulated by George et al. in 1966 which explained "excess red cell destruction" in malaria by hypersplenic activity. George and his colleagues (1966) drew on older observations by Doan (1949) and Dameshek (1955), that increased spleen size regardless of cause, was closely associated with an increased rate of red cell sequestration. The investigations of Cantrell and Elko in 1964 which demonstrated increased reticuloendothelial function of \textit{P. berghei} infected rats, were cited as supportive evidence along with the work of Gorstein and Benacerraf (1960) indicating that normal animals injected with inert colloidal particles developed hemolytic anemias the magnitude of which correlated positively with the magnitude of the increase in phagocytic activity. Both of the latter hypotheses suffer from the defect that they lack a mechanism for shutting off the autoimmune or hypersplenic function which is purportedly responsible for red cell destruction when the parasites are eliminated (Kreier, 1969). At the present time, it is fairly well established that nonparasitized red cells are destroyed during malaria. The mechanisms of red cell destruction in malaria might encompass all three hypotheses through a sequence of events in which
erythrocyte lesions are induced during malaria resulting in an altered immunologic response of the host to these damaged cells. These altered cells are then sequestered in the spleen and stimulate hypersplenism (Seed, 1969).

As the above discussion indicates, the mechanisms of tissue pathology in malarial infections are not completely understood despite enormous investigative efforts. This is due in part to investigators concentrating on studies which describe the morphology and physiology of the parasite itself rather than examining the pathological alterations of the host cell which occur during parasitization. Fine structure studies utilizing the thin-section technique have been most popular (Aikawa 1966, 1967; Aikawa and Beaudoin, 1969; Blackburn and Vinijchaikul, 1970). Such studies however are less suitable for obtaining information on cell surface alterations than carbon replication, scanning and freeze-etching. Recently, Arnold et al. (1969) utilizing scanning electron microscopic techniques reported severe morphologic alteration of the red cell surfaces in both parasitized and nonparasitized cells. Kreier et al. (1972) reported shape changes in erythrocytes of infected red cell populations. The changes correlated with parasite growth and development. The influx of abnormally shaped young cells following the anemia was in part
responsible for the morphologic changes.

The changes which occur in physiological function and chemical composition of erythrocyte membranes during the course of infection are not fully described. Changes in osmo-regulation in red cells of animals with malaria have been examined by various investigators. Dannon and Gunders (1962) reported that in rodents with malaria osmotic fragility increased as parasitemia increased. Herman (1969) reported that osmotic fragility of normal duck erythrocytes was increased by in vitro exposure to cell free extracts of *Plasmodium lophurae*. There is some evidence that loss of osmotic resistance of erythrocytes of animals with plasmodial infection is related to malfunction of ion transport systems. Overman (1948) reported abnormal permeability to sodium and potassium ions in erythrocytes obtained from malarious monkeys. Dunn (1969b) verified Overman's work and further demonstrated that even nonparasitized erythrocytes in the circulation of animals with malaria were altered.

Thus it can be seen that many workers have studied one or another facet of pathological change in erythrocytes of animals with malaria, however, few have attempted an integrated study of these changes and their correlation with the physiological changes which occur in the erythrocyte population during infection. The erythrocyte population in a healthy animal is in dynamic
equilibrium; there is a constant and steady input of newly produced erythrocytes and an equal and also steady removal of senescent ones. The population as a whole consists of erythrocytes of all possible ages in equal numbers. As infection begins the new subclass of parasitized erythrocytes appears in the population, these cells increase in number, reach a peak and then disappear. Parasitized cells also change with the growth and development of the parasites. As the infection progresses and erythrocytes are destroyed by the parasite's actions, the hematological equilibrium is upset. To compensate for cell loss input rates increase, and as a result young erythrocytes increase their representation in the population. In the later stages of infection the class of "crisis reticulocytes" appears in the population. These cells are large, commonly irregularly shaped and basophilic (Kreier et al., 1972). They have a wide range of life spans some being very short lived and some having an almost normal life span.

The animal whose erythrocyte mass has been replaced by reticulocytes of this type mimics hematologic equilibrium (Mohan 1971). The data obtained from studies of erythrocytes of animals with malaria must be interpreted with a full consciousness of the complex changes in the erythrocyte population or the interpretation is meaningless. This thesis reports an attempt at an integrated study of the changes in the erythrocyte population set in motion by plasmodial infection.
MATERIALS AND METHODS

Experimental animals and parasites: In all experiments White leghorn chickens were the host for the malaria parasite, *Plasmodium gallinaceum*. The birds were housed, fed and handled according to the standards set by the National Society for Medical Research.

The strain of *Plasmodium gallinaceum* used in this study was originally obtained from Dr. Geoffrey M. Jeffery, Institute of Malaria Chemotherapy, National Institutes of Health, Bethesda, Maryland. The infection in chickens was maintained by weekly syringe passage of infected blood.

Hematological procedures: On each blood sample collected for any experiment, packed red cell volumes (pcv) were determined by conventional methods, and percentage of red cells infected (%irbc) and percentage of basophilic erythrocytes (%brbc) present were determined by direct microscopic examination of Giemsa-stained thin blood films.

Course of infection: For purposes of experimentation and discussion, the course of the avian malarial infection was defined on the basis of the animals hema-
tological picture having six phases. They were:

1. "Normal". Preinfection state.

2. Prepatent state. This phase occurs approximately 5-7 days after infection. There are no infected cells found upon examination of thin blood films and no basophilic erythrocytes occur in the blood.

3. Early patent period. In this stage there is rising parasitemia, but less than 10% of the cells are infected and no basophilic cells are seen. This phase occurs approximately 6-9 days after infection.

4. Middle patent period. This phase of infection is characterized by high parasitemia and low basophilia (less than 10%). This phase occurs approximately 10-12 days after infection.

5. Late patent period. During this stage there is falling parasitemia and rising basophilia. It occurs approximately 11-14 days after infection.

6. Recovery state. This stage is characterized by very low or no parasitemia and falling basophilia. It occurs approximately 13-20 days after infection.

Induction of non-infectious anemias by phenylhydrazine injection or by bleeding: Anemias were induced in healthy chickens by three intramuscular injections (IM) of phenylhydrazine hydrochloride. On day one, 5 mg were injected, on day three, 10 mg, and on day five, an additional 10 mg. Anemias were also induced in birds by withdrawing large volumes (20 ml) of blood (IC) on
Phase and Normarski interference microscopy: Venous blood samples were collected in heparinized syringes. The blood samples were centrifuged (1,000 x g for 5 min.), plasma removed and the red cells gently washed twice in Ringer's solution (appendix). The washed packed erythrocytes were resuspended to a 1% (v/v) concentration. Droplets of these suspensions were placed on gelatin coated microscope slides and covered with coverslips. The slides were examined with either a phase-contrast or a Normarski interference microscope.

Bright field microscopic examination of Giemsa stained, carbon shadowed erythrocytes: One ml blood samples were collected in heparinized syringes containing 1 ml of a 1% glutaraldehyde-Ringer's solution, pH 7.3. The blood samples were fixed in the collecting syringes for 30 minute, centrifuged and the erythrocytes washed twice in Ringer's solution. After washing, the red cells were again resuspended in the fixative and incubated for another hour. The red cells were washed twice in distilled water and resuspended to a 1% (v/v) concentration. Droplets were placed on microscope slides and allowed to air dry, after which the slides were stained with phosphate-buffered (pH 7.3) Giemsa reagent. The cell preparations were lightly shadowed with carbon at about a 45° angle in a EM evaporator. Preparations were examined with a standard bright field microscope.
Electron microscopic examination of carbon replicated red cells: Red cell samples were washed and fixed by methods similar to those described previously for the preparation of carbon shadowed erythrocytes. Carbon replicas of chicken erythrocytes were prepared by a modified version of the method of Bradley and Williams (1957). The sample grids were first shadowed (20°) with platinum. Carbon was then deposited at a 45° angle in addition to shadowing from the recommended 90° angle. The replicas were prepared for electron microscopic viewing by treating the grids first with chloroform to remove the formvar coat and then with a sulfuric acid solution which contained 15% (w/v) potassium permanganate plus 15% (w/v) sodium dichromate, to remove the organic material. Acid treatment was followed by a series of distilled water rinses.

In order to locate the approximate positions of the intracellular parasites within replicated erythrocytes, red cell suspensions were placed on EM grids and allowed to air dry. The red cells were then stained with Giemsa reagent and photographed by bright field microscopy prior to replication. After replication the areas previously photographed by bright field microscopy were located with the electron microscope and again photographed.
Scanning electron microscopy in combination with bright field light microscopy: Erythrocytes were collected, fixed and washed as described for bright field microscopy of carbon shadowed preparations. The glutaraldehyde fixed red cells were resuspended to a 0.1% (v/v) concentration in distilled water. Droplets of each of the samples were placed on glass coverslips and allowed to air dry. The samples were then stained with buffered (pH 7.3) Giemsa reagent. The stained films were examined by light microscopy and selected areas photographed. The samples were then placed on a rotating table in an EM evaporator and shadowed with gold. Areas previously photographed by light microscopy were located with the scanning electron microscope and photographed.

EM thin sections examined by standard transmission electron microscopy: Blood samples were collected in heparinized syringes. The red cells were washed twice in Ringer's solution, then fixed for one hour in 1% glutaraldehyde-4% sucrose, phosphate buffer (pH 7.3). After this fixation, the samples were washed twice in 4% sucrose-phosphate buffer and fixed further in 1% osmium for 1 hour. The specimens were then dehydrated in a series of ethanol baths of increasing concentration and propylene oxide and embedded in Epon 812 (Luft, 1961). Thin sections were cut and were stained with uranyl acetate-lead citrate (Echlin, 1964).
Freeze-cleaved red cells examined by standard transmission electron microscopy: Prepared erythrocytes samples were freeze-cleaved, etched and replicated in the Balzer's apparatus according to the modified method of Moore and Mühlethaler (1963) described by Seed et.al (1971). Blood samples were drawn from healthy and *Plasmodium gallinaceum* infected chickens. The erythrocytes in the collected samples were spun down (770 x g) and washed three times in Ringer's solution. The packed red cell samples were resuspended in 20% glycerol-Ringer's solution and stored overnite at 4°C. The samples were then centrifuged and fine droplets of each of the packed cell samples was placed on copper discs with a pasteur pipetted having a small orifice. The red cell droplets were immediately frozen in liquid Freon and then transferred to liquid nitrogen. The samples were placed on a pre-cooled (-100°C) table in the etching apparatus after which the samples were cleaved and then etched for two minutes. Replicas of the fractured samples were made by shadowing with a platinum-carbon mixture. The samples were removed from the etching device and placed in distilled water. The replicas were prepared for EM examination by one hour treatments of: 70% sulfuric acid; distilled water; commercial bleach containing 14% NaClO in NaOH; and then again in distilled water.
Osmotic fragility: The osmotic properties of the red cells in each of the samples were tested by a method modified from that described by Herman (1969). Two percent suspensions of the washed red cells which contained approximately $0.12 \times 10^9 \text{rbc/ml}$ were prepared. Six tenths milliliter of the cell suspensions were pipetted into tubes of saline of decreasing tonicity, each containing 5.4 ml. The concentrations (in %) of physiological saline used were: 0.00, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, and 1.00. The tubes were first incubated at 37°C for 30 minutes and then at 4°C for 12 hours. The tubes were centrifuged at 1,300 x g for 5 minutes and the upper 2 ml of supernatant were pipetted off. The hemoglobin released into the supernatant was determined for each of the samples spectrophotometrically at a wavelength of 540 nm. The optical density of erythrocyte samples lysed in distilled water was the standard for 100% hemolysis.

For visual demonstration of differences in the osmotic resistance of normal and infected red cells, washed erythrocytes were suspended to a 1% (v/v) concentration in hypotonic (0.45% NaCl) salt solutions and incubated at room temperature for 30 minutes. Droplets were placed on microscope slides, covered with glass slips and examined by bright field microscopy with an oil immersion lens.
Red cell volume measurement: The blood was fixed in 1% (v/v) glutaraldehyde-phosphate buffered saline (pH 7.3) for 10 minutes. The fixed red cells were washed twice in physiological saline. The packed cells were diluted 1:20,000 in filtered saline. The red cell suspensions were placed in an electronic particle counter and counts were taken at 5 unit intervals starting with 10 and ending with 80. The 100 um orifice was used for all the counts. Trigger numbers, at which counting efficiency was 50%, were correlated to specific volumes obtained from a standard curve constructed by plotting threshold readings of reference spheres (1-15 um diameters) against their known volumes.

To determine red cell size and approximate cell volume by direct microscopic examination whole blood was treated as for electronic counting. Fixed erythrocytes were diluted to a 1% concentration in physiological saline. Droplets of such suspensions were placed on microscope slides and allowed to air dry. The slides were rinsed in distilled water and then stained with Giemsa reagent and examined by bright field microscopy. Individual cells were measured in length, width and depth with a calibrated ocular micrometer. The depth of these cells could be approximated since in these fixed preparations many of the clumped cells were upright as evidenced by nuclear orientation and general cell morphology. The measurements were used in determining red cell
volumes mathematically. Measured cells were grouped according to stage of infection and maturity.

*Intraerythrocytic sodium and potassium ion concentration determinations:* Alterations in intraerythrocytic ion concentrations were determined by atomic absorption spectrophotometry. A Perkin-Elmer AA spectrophotometer, model 403\textsuperscript{1} was used for all determinations. The recommended instrumentation parameters were followed for Na\textsuperscript{+} and K\textsuperscript{+} analysis (Kerber, 1971). Sodium concentrations were determined at 295\text{nm}. Potassium concentrations were analysis at 383\text{nm}.

Whole blood was collected in heparinized syringes. The blood was centrifuged at 770\text{g} for 5 minutes. The plasma and the buffy coats were removed. The packed cells were washed, centrifuged and resuspended to the volume of the original blood samples in Ringer's solution. Packed cell volumes were determined on the suspensions as were red cell numbers in the suspensions. One tenth milliliter aliquots of the red cell suspensions were pipetted into 9.9\text{ml} and 19.9\text{ml} of double-distilled, deionized water for Na\textsuperscript{+} and K\textsuperscript{+} determinations respectively. The red cells were allowed to lyse completely after which the red cell ghosts were removed by centrifugation (7,500\text{g} for 10 min). The supernatant fluids were analysed for sodium and potassium. Intraerythrocytic ion concentrations were adjusted for the ions of the suspending fluid. The volume of the suspending fluid

\textsuperscript{1} Perkin-Elmer Corp., Norwalk, Connecticut, U.S.A.
was determined by subtracting the total red cell volume, estimated by multiplying individual mean cell volume by the number of red cells per aliquot, from the total volume of the aliquots.

**Cytochemical localization of ATPase:** The cytochemical methods employed were similar to those described by Voelz and Ortigoza (1968). Whole blood collected in heparinized syringes was fixed for 30 minutes in 1% glutaraldehyde-4% sucrose, phosphate buffer, pH 7.3. The fixed cells were washed five times in a modified Ringer's solution which contained 10.0 mM MgCl₂. The red cells were then incubated at 41°C for 2 hours in the basic medium which contained the ATP substrate (5.0 mM) plus Pb(NO₃)₂ (0.5 mM). Controls lacked the substrate ATP. After incubation, the samples were washed twice in the basic medium, post-fixed in 1% osmium for one hour, dehydrated, and embedded in Epon 812 (Luft, 1961). Thin sections were cut on a Reichert ultramicrotome. Unstained sections were examined by electron microscopy.

**Quantitative biochemical analysis of red cell ATPase activity:** Erythrocytes washed once in Ringer's solution, pH 7.3 were resuspended in Ringer's solution to give a final cell concentration of 1.00 x 10⁹ rbc's/ml. One tenth milliliter quantities of these suspensions were pipetted into 1.9 ml of a modified Ringer's solution (pH 7.3) which contained 10.0 mM MgCl₂ and 2.5 mM ATP. Controls lacked either ATP or the whole cells. Ouabain
(10⁻⁴M) was added to some samples in order to determine the sensitivity of the enzyme system to the drug. After incubation at 41°C for 50 minutes, the enzymatic reaction was stopped by adding 1 ml of 10% trichloroacetic acid. The tubes were centrifuged at 1,800 x g for 10 minutes after which 1 ml of the supernatant fluid was pipetted off. Concentrations of inorganic phosphate were determined by methods outlined by Clark (1964). Enzymatic activity was computed in terms of uM of phosphate cleaved from the ATP substrate per minute per red cell.

Concentration of red cell and parasite cholesterol and phospholipids: Red cells were washed twice in 12 times their volume of Ringer's solution, pH 7.3. The washed red cells were resuspended to a 33% (v/v) concentration in Ringer's solution. Red cell concentrations were determined on aliquots of the suspensions. Hemoglobin free erythrocyte ghosts were prepared by lysing 0.1 ml (for phospholipid) or 0.5 ml (for cholesterol) of the 33% rbc suspensions in 100 times their volume of 20% physiological saline followed by distilled water lysis. The erythrocyte ghosts were collected by centrifugation at 1,500 x g for 10 minutes. Ten times their volume of a 2:1 chloroform:methanol solution was added to the packed ghosts. The suspensions were vigorously shaken and allowed to incubate at 25°C for 2 hours, then incubated for 48 hours at 4°C. Gross particulates were removed by passing the suspensions through compressed
cotton filters 0.5 centimeters thick. The samples were then washed twice with distilled water. The organic solvents were driven off by placing the samples in a 90°C water bath for several hours. The amount of organic lipid phosphorus per aliquot of red cell ghosts was determined by a modified Fiske-Subbarow method (1925) described by Dodge and Phillips (1967). Cholesterol was determined by the method of Webster (1964). Red cell concentrations were reported in picograms per red cell.

To calculate the contribution of the intracellular parasites to red cell lipids, parasite phospholipid and cholesterol concentrations were estimated. Nonparasitized blood and infected blood with more than 50% parasitized erythrocytes were collected. The red cells were washed twice in Ringer's solution. After centrifuging the suspensions at 770 x g for 5 minutes the packed cells were diluted with Ringer's solution to a 10% concentration. The erythrocytes were then passed through a continuous flow ultrasound apparatus at a flow rate of approximately 30 ml/minute at maximum ultrasonic intensity. The samples were centrifuged at 30 x g for 10 minutes. The top two thirds of the supernatant fluid, which contained mainly free nuclei and membrane contaminates, in the nonparasitized erythrocyte samples, or free nuclei, parasites and residue membrane contaminates in the parasitized erythrocyte samples, were
Pipetted off and centrifuged at 10,000xg for 10 minutes. After centrifugation the supernatants were aspirated off and discarded while the pellets were resuspended in 3 ml of physiological saline. Nuclei, or nuclei and parasites were counted using a hemocytometer. The phospholipid and cholesterol concentration of the preparations were determined by methods previously described. After adjustment for numbers of nuclei in the various preparations, the difference between lipid content of nuclei derived from control, non-infected red cells and the lipid content of nuclei in the mixed nuclei-parasite samples was assumed to be the lipid content of the parasites.

Osmotic properties of normal avian erythrocytes treated in vitro with plasma and plasma ultrafiltrates from healthy chickens or chickens with malaria: Normal, noninfected avian erythrocytes were washed and suspended in 10 times their volume or either normal or infected blood plasma and then incubated for 30 minutes at 37°C. The treated cells were then spun down, washed twice in physiological saline and resuspended to a 2% (v/v) concentration. The osmotic properties of the in vitro treated red cells were examined by the previously described multiple tube fragility test.

Normal and late patent phase blood plasma were filtered through membranes having pore sizes small
enough to retain plasma constituents larger than 50,000 (xm-10, Diaflow membranes) in molecular weight. Ultrafiltrates of normal (ncp-low) and infected plasma (icp-low) were tested for their fragility inducing capacity. The sodium and potassium cation concentrations in the various plasma fractions were determined by atomic absorption spectrophotometric methods. The pH of each of the fractions was also measured.

In vivo survival of Chromium$^{51}$ labeled, normal erythrocytes pretreated in vitro with normal and infected plasma and plasma ultrafiltrates: The survival of the plasma and plasma filtrate treated chicken rbc's was evaluated by the Cr$^{51}$ labeling technique previously described (Kreier and Leste, 1967). The red cells to be tested were washed twice in Ringer's solution and incubated for 30 minutes at 37°C in ten times their volume of pooled blood plasma from healthy chickens or chickens in the late patent phase of malaria or with osmotic fragility inducing, low molecular weight ultrafiltrates of the normal and infected plasma. Following incubation the treated cells were washed twice in Ringer's solution, labeled and then injected back into the chickens from which they were withdrawn.

The chickens were bled on the 1st, 18th, 43rd, 88th, 135th, 182nd and the 279th hour after infusion. One milliliter venous blood samples were taken at each bleeding period. The weight of each sample was determined.
The samples were diluted to 5 ml with double distilled water and placed in a Hewlett-Packard dry well gamma counter to determine their radioactivity.

The effect on red cell ATPase activity of incubation of erythrocytes in blood plasma or low molecular weight plasma ultrafiltrates: Normal, washed erythrocytes were incubated for 30 minutes at 41°C with pooled blood plasma or plasma ultrafiltrates from healthy chickens and chickens in the middle and late patent periods of Plasmodium infection. Incubating fluids were aspirated off and red cell concentrations were adjusted to 1.00 x 10^9 rbc's/ml in Ringer's solution. Each of the red cell samples were then assayed for ATPase by procedures described in the section on red cell ATPase activity.

The effect of trypsinization on normal red cell osmotic fragility and total ATPase activity: Aliquots of normal, washed erythrocytes from healthy chickens were incubated in 10 times their volume of a 0.25% (w/v) trypsin-Ringer's solution for 30 minutes at 41°C. The treated red cells were washed once in Ringer's solution and then tested for change in osmotic properties or assayed for total cell ATPase activity. Alteration in enzyme activity or osmotic fragility of the treated red cell samples were compared to control erythrocyte samples incubated in Ringer's solution alone.
Blood plasma chemistry prior to and during *P. gallinaceum* infection: Sodium and potassium cation concentrations in blood plasma were determined by methods described by Kerber (1971) in the Perkin-Elmer AA spectrophotometer (model 403) methods manual.

Plasma chloride concentrations were determined by electronic titration with a Cotlove Chloridometer according to standard methods (Cotlove et al., 1958).

Blood plasma concentrations of acid soluble, inorganic phosphate were determined by assaying deproteinized samples for inorganic phosphate by methods outlined by Clark (1964). For this assay, five tenths ml of plasma was diluted with 1.5 ml of distilled water and then 1 ml of 10% (w/v) trichloroacetic acid was added. The suspensions were shaken and allowed to stand for 10 minutes. The samples were centrifuged for 10 min at 1,200 x g. One ml portions of each supernatant were pipetted off and analyzed for phosphate.

The pH of blood plasma was determined on blood samples obtained by venous puncture. Blood samples were immediately chilled to 4°C and centrifuged to separate plasma from red cells. Plasma supernatants were promptly pipetted off and stored at -20°C in small volume vials capped with rubber stoppers. At the time of testing, the vials were removed from the refrigerator and thawed in such a manner as to maintain the plasma in a chilled state.
Lipids were extracted from one tenth ml quantities of plasma by the same method used to extract lipid from red cells. Lipid phosphorus was assayed by the modified method of Fiske and Subbarow (1925) described by Dodge and Phillips (1967). Blood plasma cholesterol concentrations were determined by Webster's method (1964).

Plasma samples were assayed for total protein by standard Biuret methods (Clark, 1964).

UV absorption properties of diluted blood plasma diluted 200 fold in distilled water were determined spectrophotometrically in the UV range at wave lengths of 260 nm and 280 nm. Ratios of the absorbancies at 280 nm and 260 nm were calculated.

Urea nitrogen (bun) concentrations in pooled plasma samples were assayed by the methods described by Crocker (1967).

Pooled plasma samples were assayed for blood sugar by the Harleco micro glucose, o-toluidine method (Relander 1963).

ATPase activity of plasma was determined by the procedure used in determination of red cell ATPase activity, excepting that 0.1 ml of plasma replaced 0.1 ml of an rbc suspension as the "enzyme preparation".
RESULTS

Morphology: The morphology of red cells of chickens undergoing plasmodial infection changed progressively. Large numbers of abnormally shaped red cells occurred in the population when parasitemia was high. Many morphologically abnormal red cells were also present in blood samples collected during the period when the chickens were recovering from the infection.

Figure 2 is a series of photomicrographs which depict the morphological changes which occur in erythrocytes during avian malarial infection. In the left hand, vertical column (Fig. 2A, D, G, J, M) are photomicrographs made by Kormarski interference microscopy, in the middle column (Fig. 2B, E, H, K, N) are photomicrographs made by phase-contrast microscopy and in the right hand column (Fig. 2C, F, I, L, O) are photomicrographs of Giemsa stained carbon shadowed red cell preparations. Normal, noninfected red cells are oblong in shape and resemble flattened footballs (Fig. 2A, B, C). By all three light microscopic techniques the normal red cells can be seen to be regular in size and shape. The apparent protrusion of the nuclei (n) of the erythrocytes (Fig. 2A & B) is an optical artifact. Such artifacts and other similar ones result from the optical
Figure 2. Photomicrographs of red blood cells obtained from chickens prior to infection (A,B,C) and during the prepatent (D,E,F), middle patent (G,H,I), late patent (J,K,L) and recovery phases of Plasmodium gallinaceum infection (M,N,O). The left hand vertical column is a series of photomicrographs taken by interference light microscopy; the middle column by phase-contrast; and the right hand column by bright field microscopy. Abbreviations: n, red cell nucleus; mf, myelin filaments; ip, intraerythrocytic plasmodia; bp, basophilic red cells. Line marker equals 10 um.
systems of the phase and interference microscopes which translate density differences into apparent elevation differences. That the nuclei of normal, noninfected red cells do not protrude above the plane of the surface of the erythrocytes is apparent in the carbon shadowed, Giemsa stained cell preparations shown (Fig. 2C). During the middle patent phase of infection (Fig. 2G-H) many of the cells are parasitized and appear by phase and interference microscopy to have deep surface depressions where the intracellular parasites are located (Fig. 2G,H; IP). These depressions are in part optical artifacts due to density differences between the parasites and the cytoplasm of the host cell. In Figure 2I, which is a bright field light photomicrograph of a highly parasitized red cell sample that has been fixed and carbon shadowed, the depressions are less prominent than in the phase and interference preparations.

Filamentous structures (mf) extending from the erythrocytes are seen in Figures 2G and H. Similar structures do not occur in the fixed carbon shadowed preparations (Fig. 2I). These structures may be artifacts induced during preparation of the erythrocytes. Erythrocytes examined by phase or interference microscopy are not fixed and thus may be easily distorted during microscopy. Because of the osmotic fragility of the red cells obtained from birds in the patent phases of infection, these cells are more easily altered.
in vitro than are normal, noninfected red cells treated similarly.

Many of the shape alterations which can be seen in erythrocyte preparations by these three light microscopic techniques are probably true representations of changes which occur in the host during infection. In the prepatent phase of infection very little abnormality in morphology of the red cells occurs (Fig. 2D-F). The patent period is characterized by a heterogeneous red cell population of varied morphologic types. Red cells which appear quite normal in size and shape are present. Some red cells with grossly normal shape have altered surface characteristics. Many cells appear not to have a full hemoglobin content. They appear sunken in and there are shadows associated with the nuclei (Fig. 2I & L). The percentage of abnormal erythrocytes increases greatly in the blood of infected birds in the late patent phase of the infection (Fig. 2G). The majority of cells present in the patent phase are swollen and have distorted surfaces (Fig. 2L). In the recovery phase (Fig. 2M-0) the majority of red cells appear almost normal in size and shape. They do not appear to be as "full" as the preinfection erythrocytes however and may be hypochromic (Fig. 2O).

Normal, noninfected red cells from healthy chickens disperse evenly when washed and placed on microscope
slides; red cells from chickens in the patent phase of infection clump (Fig. 2G). This is evident in the interference photomicrographs (Fig. 2A, D, G, J, M). The changes in dispersability indicate that these is membrane alteration and reduction of erythrocyte surface charge during the late patent period of malaria.

Carbon replicas of red cells obtained from chickens in the various phases of infection are shown in Figure 3. Figure 3A is a electronmicrograph of replicas of normal, noninfected erythrocytes obtained from healthy chickens. The cells are oval in shape and have smooth surfaces. No elevation of the cell surfaces in the nuclear region occurs. The cells have a fairly uniform size and shape. Replicas of erythrocytes from birds in the prepatent phase of malaria are shown in Figure 3B. A small percentage of red cells have lost some of their hemoglobin and are partially collapsed so that the nuclei stand out (Fig. 3B; arrows).

When a high percentage of the erythrocytes are parasitized, the morphology of a large percentage of the red cells is abnormal (Fig. 3C). Some erythrocytes lose copious amounts of hemoglobin and collapse (Fig. 3C, arrow-a). Many of the cells have roughened and pitted surfaces (arrows-b). Even though the percentage of red cells with surface alterations is quite high, some cells appear to have normal morphology (Fig. 3C, arrows-c).
Figure 3. Electron micrographs of carbon replicas of erythrocytes from chickens: (A) replicas of erythrocytes from a healthy chicken; (B) red cells from a chicken in the early patent phase of infection; (C) red cells from a chicken in the middle patent phase of malaria; (D) red cells from a chicken recovering from malaria, parasitemia is absent but approximately 47% of the erythrocytes are basophiles. Abbreviations: bp, basophilic erythrocyte; lec, leucocyte; arrows, rbc with collapsed surface and nucleus is prominent; arrows–a, rbc with copious amounts of hemoglobin lost; arrows–b, rbc with grossly normal shape but roughened and irregular surface; arrows–c; normal appearing rbc within an infected population. Line marker equals 10 um.
The basic oval, football shaped cell still occurs in the blood during this phase while only a very few spherocytic red cells occur.

The morphology of most of the red cells in the blood of birds in the late patent stage of malaria is decidedly different from that of normal erythrocytes. A high proportion of red cells are spherocytic (Fig. 3D). Most erythrocytes present during the recovery stage have rough and irregular surfaces. Morphologic changes occur in almost all the erythrocytes of the population. Many of the abnormal red cells are of the basophilic type (Fig. 3D;b) produced and released by hemopoietic tissues functioning under the stress caused by the disease.

The light microscopy and carbon replica EM studies described so far have shown there is change in red cell morphology during avian malaria. However, the relationship of parasitization to the erythrocyte pathology could not be determined from these studies. In order to correlate erythrocyte pathology and parasitization, a coupling of morphologic techniques was utilized. Selected blood samples from birds in the patent phase of malaria were prepared and examined first by light microscopy and then by electron microscopy. Figure 4 shows matched light (inserts) and electron micrographs. Electron micrographs A and B are of two noninfected red cells. These cells have almost normal morphology except that they have lost
Figure 4. Electron micrographs of carbon replicas of erythrocytes from *P. gallinaceum*-infected chickens showing the relationship of parasitization to erythrocyte pathology. Electron micrographs (A) and (B) are of nonparasitized erythrocytes from an infected chicken. Photographs (C) through (H) show the relationship between red cell alteration and the size of the intracellular parasite. Arrows indicate the location of the intracellular parasite in the light (inserts) and electron micrographs. The red cells in Figures (C) and (D) have small parasites. Note the slit-like lesions in the area of the intraerythrocytic plasmodia (C,D; arrows). Except for the latter, these cells appear quite normal and have little surface alteration. In contrast, the red cells in Figures (G,H and I) contain large parasites or have multiple infections. These cells are swollen and have considerable surface alteration. Electron micrograph (J) is of an abnormally shaped, non-infected red cell from a late patent phase blood sample. Line marker equals 10 μm.
hemoglobin because they are somewhat collapsed and their nuclei are evident. Parasite growth is apparently a major factor in the alteration of red cell morphology. Red cells in Figures 4C and D are parasitized by small plasmodia. Little cell surface or cell shape change from normal is present. However, there do appear to be small "slit-like" lesions on the cell surface near the intraerythrocytic parasite in a high percentage of infected erythrocytes (Fig. 4C & 4D; arrows). As the intracellular parasites grow at the expense of the host cell, surface alterations develop in the erythrocyte. Depressions and pitting are common (Fig. 4E&F). Erythrocytes containing large plasmodia are often swollen and have rough and irregular membranes (Fig. 4G&H). Sphering of parasitized red cells occurs commonly in the late patent period of infection (Fig. 4I).

The morphology of red cells collected during the late patent stage of infection is very different than that of red cells from normal, healthy chickens. During this stage of avian malaria even noninfected erythrocytes have unusual morphology (Fig.5A,4J). The direct relationship between parasitization and abnormal red cell morphology is not as distinct late in the infection as it is in the prepatent or early patent phases of infection. A large percentage of the abnormally shaped cells are nonparasitized "stress" reticulocytes. Similar abnormal
Figure 5. The morphology of carbon replicated red cells of a chicken recovering from malaria (A) and in blood of a chicken suffering from a drug induced, non-infectious anemia (B). Irregularities in shape and membrane structure of a similar type can be seen in either condition. Arrows match identical cells examined first by light microscopy (inserts) and then by electron microscopy. Line marker equals 10 um.
red cells may be seen in chickens suffering from drug-induced non-infectious anemias (Fig. 5B).

The relationships between red cell morphology and parasitization just described have been verified by scanning electron microscopy. By scanning microscopy normal erythrocytes appear as they do by the carbon replica method. They have smooth membranes; they are oval and their nuclei do not protrude (Fig. 6A). When parasitemia is high erythrocyte surface alterations commonly occur. The degree of cell alteration is usually directly related to the size of the intraerythrocytic parasite (Fig. 6B).

During the late patent period noninfected, nonbasophilic red cells often have abnormal morphology (Fig. 6C). An infected erythrocyte (arrow) and a noninfected one (Fig. 6C) both have similar abnormal shapes. However, the infected cell has membrane alteration in the location of the parasite (arrow).

Electron microscopic examination of freeze-cleaved infected red cells indicate that fully developed parasites reside in close proximity to the membrane of the host cell. Figure 7B a three dimensional, cross sectional view of an infected erythrocyte, clearly shows an intracellular malarial parasite with its inner and outer limiting membranes in extremely close proximity to the cytoplasmic membrane of the host cell. As was
Figure 6. Scanning electron micrographs of erythrocytes from a healthy chicken (A) and from infected chickens (B,C,D). Red cells shown in Figure B were obtained from a malarious chicken during peak parasitemia. Cells shown in C and D are from animals in the late patent phase of infection. Red cells from the middle patent phase of infection (Fig. B) have surface depressions in the location of the larger intracellular parasites (arrows). Abnormal cell shape is noted in both infected (arrows) and non-infected erythrocytes (Figures C,D). Arrows indicate location of intracellular plasmodia. Line marker equals 10 um.
Figure 7. Partial cross sectional views of freeze-cleaved erythrocytes. Electron micrograph (A) is of a normal red cell from a healthy chicken. Nuclear ultrastructure and cytoplasmic organelles are evident. Limiting cytoplasmic membrane is shown. Figure B is a micrograph of a plasmodia infected red cell. An intra-erythrocytic parasite (P), with its limiting membranes, is shown. Note the close proximity of the parasite to the limiting plasma membrane. Abbreviations: CM, cytoplasmic membrane; N, host cell nucleus; NM, nuclear membrane; G, golgi apparatus; HB, hemoglobin; P, plasmodia; ILM, inner limiting membrane of the parasite; OLM, outer limiting membrane of the parasite. Line marker equals 1 um.
Figure 8. Electron micrographs of thin-sectioned erythrocytes. Figure A is a high power micrograph of a normal, non-infected red cell. A uniform limiting plasma membrane is noted and is characteristic of red cell membranes of healthy chickens. Electron micrograph B shows an intracellular parasite (P) closely associated with the limiting host cell membrane (CM). Irregularities are present in the cytoplasmic membrane (MC) of the parasitized cell. Abbreviations: CM, cytoplasmic membrane; HN, host cell nucleus; P, parasite; PM, parasite's limiting membranes; MC, microcrenation. Line marker equals 1 um.
noted in the previously described carbon replica and scanning microscopy studies, the red cells with altered surfaces usually contain the larger parasites. As the malarial parasites grow they come into close association with the limiting cytoplasmic membranes of the host red cells. This association results in distortion of host cell membranes. An electron micrograph of a thinly sectioned infected red cell (Fig. 8B) also shows the close relationship of the parasite to the host cell membrane. What appears to be the start of microcrenation formation (MC) is evident in this micrograph (Fig. 8B). Such surface alterations are not seen in micrographs of thin sections of noninfected red cells of healthy chickens (Fig. 8A).

**Osmotic Fragility:** Erythrocytes taken during the patent phase of infection from *Plasmodium*-infected chickens had altered osmotic properties. These red cells were quite fragile and did not withstand as much osmotic stress as did normal erythrocytes from healthy chickens. (Table 1). The fragility increased in proportion to the rise in parasitemia. The percent hemolysis of maximally parasitized red cells incubated in the 30% and 40% hypotonic saline solutions was such that one must conclude that the percentage of osmotically resistant cells within the population was less than normal. (Table 1). As the birds began to recover
Table 1. Osmotic resistance of red cells obtained from chickens prior to and during the course of *Plasmodium gallinaceum* infection. The data indicates that during stages of infection when parasitemia is rising the percentage of erythrocytes lysed is proportional to the percentage of parasitized red cells within the sample. Erythrocytes obtained from chickens late in the patent period of infection have significantly elevated osmotic fragilities which are disproportionate to parasitemia.
<table>
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<tr>
<th>DAY</th>
<th>In: 50% saline/d-H₂O</th>
<th>40%</th>
<th>30%</th>
<th>PCV</th>
<th>%IRBC</th>
<th>%RBBC</th>
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<td>preinfection</td>
<td>38.7 ± 6.3</td>
<td>77.7 ± 4.0</td>
<td>93.3 ± 1.4</td>
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<td>61.0 ± 5.8</td>
<td>88.6 ± 2.3</td>
<td>30.8</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>35.6 ± 5.9</td>
<td>79.0 ± 4.3</td>
<td>94.4 ± 1.7</td>
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<td>1.1</td>
<td>0</td>
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<tr>
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<td>72.1 ± 8.4</td>
<td>91.1 ± 3.7</td>
<td>98.2 ± 1.1</td>
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<td>25.2</td>
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<td>88.2 ± 3.5</td>
<td>100.0 ± 0.0</td>
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<td>37.1</td>
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<td>30.1</td>
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* = not statistically significant at the 95% confidence level. Student's "t" test.

s = statistically significant at the 95% confidence level. Student's "t" test.

* = mean value ± standard error.
red cell fragility decreased from the levels which occurred during patency but fragility was still elevated above normal. In blood samples from recovering birds, red cell fragility did not correlate with the parasitemia (Table 1; day 17). During the recovery period when the hematological picture of the bird appeared normal (Table 1; day 19), its erythrocytes exhibited similar osmotic resistance to hypotonic lysis in 50% physiological saline as red cells from healthy chickens. In 30% or 40% physiological saline these cell samples (day 19) were more resistant to lysis than normal cell samples. The increased osmotic resistance of these red cell samples was probably due to the high percentage of young erythrocytes within the blood during the recovery phase.

During the late patent phase of infection the non-parasitized red cells as well as parasitized ones had altered osmotic properties. Figure 9A shows normal red cells osmotically stressed by incubation in a hypotonic salt solution. The cells are slightly spherocytic. A low percentage of the erythrocytes are lysed. In contrast, similarly treated erythrocytes from a chicken in the late patent period of infection are all spherocytic (Fig. 9B). A high percentage of cells are lysed. As many nonparasitized erythrocytes as parasitized ones are swollen or lysed.
Figure 9. Phase-contrast photomicrographs of normal erythrocytes (A) and erythrocytes from a *Plasmodium gallinaceum* infected chicken in the late patent phase of infection (B) suspended in hypotonic salt solution. The red cells from the infected chicken (B) are more fragile than the red cells from a healthy chicken (A).

Abbreviations: SRBC, spherocytic red blood cell; LIC, lysed infected cell; LNC, lysed non-infected cell. Line marker equals 10 μm.
Prolonged periods of hematological stress regardless of cause induce fragile red cell populations in animals. Even noninfected chickens stressed by extensive bleeding or by injection of hemolytic drugs have osmotically fragile (Fig. 10) and morphologically abnormal red cell populations (Fig. 5B).

**Erythrocyte volume:** The changes in osmotic fragility of erythrocytes from chickens with *Plasmodium gallinaceum* infection correlated with erythrocyte volume changes. The most fragile erythrocytes had large cell volumes. Figure 11 shows the volumes of red cells obtained from birds prior to *P. gallinaceum* infection and during the middle and late patent phases of the disease as determined by electronic cell sizing. A high percentage of the normal, preinfected red cell population fell within a narrow range of volumes. With initiation of infection and the onset of patency, there developed a wide distribution of erythrocyte volumes about the mean. The mean red cell volume (mcv) also increased, from approximately 90 um³ prior to infection to 110 um³ at peak parasitemia. The greatest average red cell volume (~125 um³) occurred during the late patent phase of infection. Erythrocyte volumes were also large in birds which recently recovered from plasmodial infection in which parasitemia was negligible (Table 2). The
Figure 10. The osmotic fragility of red cells obtained from animals stressed by extensive bleeding (post-bleed) or by injection of the hemolytic drug, phenylhydrazine (post-phenyl.). The fragility curves shift to the right following treatment indicating a decrease in osmotic resistance of the sampled cells to the stress of incubation in hypotonic salt solutions. Post-treatment blood samples are characterized by reduced packed red cells volumes and elevated basophilic erythrocyte counts.
Figure 11. Red cell volume distributions, determined by an electronic particle counting and sizing device, of erythrocytes in samples from normal chickens (pre-infection) and chickens in the middle (peak) and late patent (post-peak) phases of malaria. Mean cell volumes increase and cell volume distribution about the mean spreads with the onset of patent infection.
Table 2. Mean red cell volumes in chickens with *Plasmodium gallinaceum* infections. Cell volumes were determined by an electronic particle counting and cell volume-sizing device.
Table 2.

<table>
<thead>
<tr>
<th>STAGE OF INFECTION</th>
<th>MEAN RED CELL VOLUME (μm^3)</th>
<th>HEMATOLOGY</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pocv</td>
<td>%rbc</td>
</tr>
<tr>
<td>normal (preinfection)</td>
<td>88.7 ± 1.20</td>
<td>30.2</td>
<td>0.0</td>
</tr>
<tr>
<td>prepatent</td>
<td>94.8 ± 3.80</td>
<td>27.5</td>
<td>0.0</td>
</tr>
<tr>
<td>early patent</td>
<td>97.3 ± 3.32</td>
<td>26.3</td>
<td>4.2</td>
</tr>
<tr>
<td>middle patent</td>
<td>109.4 ± 3.91</td>
<td>20.6</td>
<td>29.2</td>
</tr>
<tr>
<td>late patent-1</td>
<td>122.8 ± 6.65</td>
<td>15.2</td>
<td>14.6</td>
</tr>
<tr>
<td>late patent-2</td>
<td>123.7 ± 4.54</td>
<td>19.9</td>
<td>0.0</td>
</tr>
<tr>
<td>recovery</td>
<td>99.4 ± 2.96</td>
<td>26.4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* = mean value ± standard error.
ns = not significantly different from normal sample at 95% confidence level.
s = significantly different from normal sample at 95% confidence level.
(n) = number sampled.
mean red cell volumes determined by electronic cell sizing and by direct microscopic examination were very similar (Table 3). By direct microscopic examination the average normal red cell from a healthy chicken measured about 10.3 um x 5.6 um x 1.9 um and had a volume of about 90 um³. During the late patent period of infection the large basophilic erythrocyte was the cell type which contributed the most to the increase in red cell volume. This cell type had a volume of about 148 um³ and was considerably larger than the other cells found in blood of chickens in the late patent phase of infection. Red cells of all types, however, including noninfected, nonbasophilic erythrocytes within the late patent phase blood samples had larger volumes than those from healthy chickens. Parasitized erythrocytes collected at any stage of infection were considerably longer (10.6 um - 10.9 um) and narrower (5.1 um - 5.3 um) with greater thickness than normal erythrocytes (2.2 um - 2.3 um). The degree of distortion was greater in erythrocytes with larger parasites than in erythrocytes with smaller ones (Table 3; Fig. 4). During the late recovery period the red cells again had smaller dimensions with decreased cell volumes (Table 2 & 3) as compared to erythrocytes from acutely infected chickens (Table 3).
Table 3. The relationship of parasitization to red cell size and volume as determined by direct microscopic examination in chickens with *Plasmodium gallinaceum* infection.
<table>
<thead>
<tr>
<th>SAMPLE TYPE</th>
<th>SIZE-VOLUME RELATIONSHIPS</th>
<th></th>
<th></th>
<th>HEMATOLOGY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*size (um)</td>
<td>estimated volume (um³)</td>
<td>(n)</td>
<td>pcv</td>
</tr>
<tr>
<td>normal (preinfection)</td>
<td>10.3x5.6x1.9</td>
<td>90.5</td>
<td>70</td>
<td>30.2</td>
</tr>
<tr>
<td>prepatent</td>
<td>9.9x5.7x1.9</td>
<td>94.0</td>
<td>100</td>
<td>32.1</td>
</tr>
<tr>
<td>early patent</td>
<td>10.0x5.5x1.9</td>
<td>92.5</td>
<td>50</td>
<td>32.7</td>
</tr>
<tr>
<td>middle patent</td>
<td>10.1x5.2x2.2</td>
<td>96.5</td>
<td>150</td>
<td>25.2</td>
</tr>
<tr>
<td>late patent</td>
<td>10.0x5.7x2.5</td>
<td>125.0</td>
<td>125</td>
<td>18.4</td>
</tr>
<tr>
<td>cell type:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) small parasite</td>
<td>10.7x5.3x2.2</td>
<td>112.0</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>2) medium</td>
<td>10.6x5.3x2.2</td>
<td>111.0</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>3) large</td>
<td>10.9x5.1x2.3</td>
<td>115.0</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>4) basophile-1</td>
<td>9.3x5.6x2.4</td>
<td>109.0</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>5) basophile-2</td>
<td>10.4x7.0x2.4</td>
<td>114.0</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>6) non-infected</td>
<td>10.6x5.7x2.2</td>
<td>118.0</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>recovery</td>
<td>10.3x5.7x2.2</td>
<td>111.0</td>
<td>70</td>
<td>28.7</td>
</tr>
</tbody>
</table>

* mean value at n number determinations.
Intraerythrocytic sodium and potassium concentrations:

Red cells from healthy chickens prior to infection contained high concentrations of potassium ($5.59 \times 10^{-13}$ g $K^+$/rbc or about 80 meq/ml packed rbc's) and low concentrations of sodium ($0.916 \times 10^{-13}$ g $Na^+$/rbc or about 20 meq/ml packed rbc's) (Table 4). During the prepatent and early patent period of infection potassium levels decreased whereas sodium concentrations increased. Intraerythrocytic sodium concentrations reached a maximal level during the late patent phase of infection at which time there was approximately a five fold increase over normal. Cell sodium concentration decreased during the recovery phase, however, the sodium levels at this time were still higher than in normal erythrocytes. Following the drop in red cell potassium concentration during the early phases of infection, potassium concentrations increased. The increase started when basophilic erythrocytes appeared in the circulation. Red cell samples with high potassium levels from chickens recovering from malaria had a high percentage of basophilic erythrocytes. The preinfection $K^+/Na^+$ ratio (6:1) dropped to 2.4:1 during the prepatent period and continued to fall throughout the patent period. The lowest cation ratio 1.6:1 occurred following peak parasitemia. In the recovery period cation ratios slowly returned to normal.
Table 4. Sodium and potassium concentrations determined by atomic absorption spectrophotometry of erythrocytes of chickens with avian malaria.
Table 4.

<table>
<thead>
<tr>
<th>STAGE OF INFECTION</th>
<th>( \text{Na}^+ \text{ per Red Cell} ) (( \times 10^{-13} \text{gr Na}^+/\text{rbc} ))</th>
<th>( \text{K}^+ \text{ per Red Cell} ) (( \times 10^{-13} \text{gr K}^+/\text{rbc} ))</th>
<th>HEMATOLOGY ( \text{pcv}, % \text{irbc}, % \text{brbc} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal (preinfection)</td>
<td>( *0.916 \pm 0.088 ) (22)</td>
<td>( 5.590 \pm 0.120 ) (28)</td>
<td>32.9 0.0 0.0</td>
</tr>
<tr>
<td>prepatent</td>
<td>( 2.082 \pm 0.143 ) (21) s</td>
<td>( 4.960 \pm 0.156 ) (21) s</td>
<td>32.0 0.0 0.0</td>
</tr>
<tr>
<td>early patent</td>
<td>( 6.823 \pm 0.230 ) (14) s</td>
<td>( 5.070 \pm 0.201 ) (16) s</td>
<td>29.0 2.3 0.6</td>
</tr>
<tr>
<td>middle patent</td>
<td>( 2.606 \pm 0.350 ) (10) s</td>
<td>( 5.570 \pm 0.302 ) (11) ns</td>
<td>23.1 30.4 2.0</td>
</tr>
<tr>
<td>late patent</td>
<td>( 4.624 \pm 0.762 ) (10) s</td>
<td>( 6.710 \pm 0.150 ) (17) s</td>
<td>18.1 9.3 34.8</td>
</tr>
<tr>
<td>recovery</td>
<td>( 2.790 \pm 0.211 ) (14) s</td>
<td>( 6.250 \pm 0.293 ) (16) s</td>
<td>27.2 0.1 4.8</td>
</tr>
</tbody>
</table>

* = mean value \pm standard error.

s = significantly different value from normal at 95% confidence level.

ns = not significantly different value from normal at 95% confidence level. Student's "t" test.

(n) = number sampled.
The alterations in concentration of intracellular sodium and potassium during avian malaria were mimicked in birds injected with phenylhydrazine (Table 5). On the day following drug administration a two and one half fold increase in cell sodium occurred, but potassium concentration remained at near normal levels. When drug injection was continued the potassium concentration increased as did the percentage of basophilic erythrocytes in the blood.

**Erythrocyte ATPase activity:** Cytochemical studies indicated that the major sites of ATPase activity were in the limiting plasma membrane (Fig. 12A, CMA). Activity was greater in general in membranes of normal chickens than of infected chickens. Nuclear membranes had low enzymatic activity (Fig. 12D, NMA). Some ATP hydrolysis also occurred in the chromatin (Fig. 12D; na). Red cell cytoplasm generally lacked activity except for occasional active "spherical body inclusions" which lay close to the nucleus. The ATPase activity of intraerythrocytic plasmodia varied depending on the stage of maturation (Fig. 12C,D).

By quantitative biochemical methods it was determined that normal erythrocytes from healthy chickens had ATP hydrolytic activity equivalent to $1.311 \times 10^{-10}$ um Pi/min/rbc (Table 6). The enzymatic activity was partly sensitive to ouabain (g-strophanthin). About 10% decrease in activity was noted when red cells from
Table 5. Sodium and potassium concentrations determined by atomic absorption spectrophotometry of erythrocytes of chickens injected with phenylhydrazine.
<table>
<thead>
<tr>
<th>DAY POST-INJECTION</th>
<th>AMT. DRUG GIVEN PHENYLHYDRAZINE</th>
<th>Na⁺ per RED CELL (x10⁻¹³ gr Na⁺/rbc)</th>
<th>K⁺ per RED CELL (x10⁻¹³ gr K⁺/rbc)</th>
<th>HEMATOLOGY pcv %brbc</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>1.003 ± 0.096 (25)</td>
<td>5.468 ± 0.042 (31)</td>
<td>32.9 0.0</td>
</tr>
<tr>
<td>1</td>
<td>5 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>2.507 ± 0.797 (3)</td>
<td>5.643 ± 1.800 (3)</td>
<td>22.3 10.3</td>
</tr>
<tr>
<td>3</td>
<td>10 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>2.707 ± 0.108 (3)</td>
<td>7.490 ± 0.172 (3)</td>
<td>26.8 23.0</td>
</tr>
<tr>
<td>5</td>
<td>10 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>1.533 ± 0.061 (3)</td>
<td>7.293 ± 0.187 (3)</td>
<td>27.1 29.7</td>
</tr>
</tbody>
</table>

* = mean value ± standard error.  
(n) = number sampled.
Figure 12. Localization of ATPase activity by cytochemical methods. Figure 12A is an electron micrograph of a thin section of normal chicken erythrocytes incubated with ATP substrate and lead nitrate prior to embedding. At sites of ATPase activity, electron dense lead salts have been deposited. Limiting plasma membranes have high ATPase activity whereas nuclear membranes have little. Figure B, C and D are electron micrographs of erythrocytes obtained from a chicken with malaria. The plasma membranes of erythrocytes from infected birds have less enzyme activity than normal erythrocytes from healthy chickens. Differences in ATPase activity within various plasmodia occur.

Abbreviations: HN, host cell nucleus; hb, hemoglobin; P, intraerythrocytic plasmodia; na, nuclear ATPase activity; nma, ATPase activity in nuclear membranes; CMA, cytoplasmic membrane activity. Line markers equals 1 um.
healthy chickens were incubated with the cardiotyco-
side at $10^{-4}$M concentration. Erythrocytes from
Plasmodium-infected birds were inhibited to a lesser
extent ($>5\%$) at the same molar concentration of oubain.

In chickens with malaria (Table 6) erythrocytes
ATPase activity decreased slightly during the prepatent
period. Enzymatic activity was depressed at the time of
onset of patent blood infection and remained depressed
well into the late patent phase of the disease. The
lowest level of cell enzyme activity, $0.650 \times 10^{-5}$M Pi
min/rbc, occurred shortly after peak parasitemia.
Erythrocytes collected from chickens in the late patent
and in the recovery periods, when the major portion
of the cell population was immature erythrocytes
and young normacytes had enzyme activity greater than
that of normal red cells from healthy chickens.

Some insight of the relationship of red cell ATPase
activity to the stress of hemolysis induced by malaria
was given by study of ATPase activity in red cells of
chickens made anemic by injection on phenylhydrazine
(Table 7). Following the initial injection of
phenylhydrazine, when birds were anemic and had not
yet compensated for extensive red cell loss, their
red cells had significantly lower ATPase activity than
normal cells. Later as the birds adjusted to the hemo-
lytic stress by increasing cell production and releasing
Table 6. Red cell ATPase activity in erythrocytes of chickens with *Plasmodium gallinaceum* infection.
Table 6.

<table>
<thead>
<tr>
<th>STAGE</th>
<th>DAY</th>
<th>ATPase ACTIVITY OF WHOLE RED CELLS (x 10^-10 μM Pi/min/rbc)</th>
<th>HEMATOLOGY</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>pcv</td>
<td>%rbc</td>
</tr>
<tr>
<td>normal</td>
<td>0</td>
<td>* 1.311 ± .069</td>
<td>33.5</td>
<td>0.0</td>
</tr>
<tr>
<td>prepatent</td>
<td>4</td>
<td>1.155 ± .108</td>
<td>33.6</td>
<td>0.0</td>
</tr>
<tr>
<td>early patent</td>
<td>6</td>
<td>0.753 ± .077</td>
<td>32.1</td>
<td>1.6</td>
</tr>
<tr>
<td>middle patent</td>
<td>8</td>
<td>0.771 ± .131</td>
<td>22.1</td>
<td>30.5</td>
</tr>
<tr>
<td>late patent-1</td>
<td>10</td>
<td>0.630 ± .057</td>
<td>15.5</td>
<td>22.6</td>
</tr>
<tr>
<td>late patent-2</td>
<td>12</td>
<td>1.565 ± .249</td>
<td>21.5</td>
<td>7.6</td>
</tr>
<tr>
<td>recovery</td>
<td>14</td>
<td>1.634 ± .330</td>
<td>27.8</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* = mean value ± standard error.
ns = not significantly different from normal value at 95% confidence level.
s = significantly different from normal at 95% confidence level.
(n) = number sampled
Table 7. The relationship of hemolytic crisis and recovery to ATPase activity in erythrocytes of chickens injected with phenylhydrazine.
Table 7.

<table>
<thead>
<tr>
<th>DAY POST-INJECTION</th>
<th>AMT. DRUG GIVEN phenylhydrazine (IM)</th>
<th>ATPase ACTIVITY ( \times 10^{-10} \mu M \text{ Pi/min/rbc} )</th>
<th>HEMATOLOGY pcv %brbc</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>1.100 + .099</td>
<td>34.3</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>10 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>1.195 + .094 ( s )</td>
<td>29.4</td>
<td>6.3</td>
</tr>
<tr>
<td>3</td>
<td>20 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>0.665 + .046 ( s )</td>
<td>25.2</td>
<td>39.0</td>
</tr>
<tr>
<td>5</td>
<td>30 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>1.008 + .082 ( s )</td>
<td>24.5</td>
<td>47.5</td>
</tr>
<tr>
<td>7</td>
<td>40 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>2.066 + .059 ( s )</td>
<td>26.6</td>
<td>38.3</td>
</tr>
<tr>
<td>9</td>
<td>0 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>3.164 + .265 ( s )</td>
<td>31.5</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* = mean value + standard error.
\( s \) = value significantly different from normal at 95% confidence level.
immature red cells from hemopoietic tissues ATPase activity increased. During this period red cell ATPase activity increased to normal levels or above (1.100 x 10^{-10} \text{uM Pi min/rbc} to 3.160 x 10^{-10} \text{uM Pi/ min/rbc}).

**Erythrocyte lipids:** Total erythrocyte phospholipid concentrations increased with the onset of active blood infection. Normal erythrocytes had 0.550 picograms of phospholipid per erythrocyte (Table 8). At peak parasitemia phospholipid concentrations were greatly elevated (1.166 picograms per cell). High levels of phospholipid (1.198 picograms per cell) also occurred in erythrocytes from chickens in the late patent phase of infection when few parasitized cells were present but when basophilic erythrocytes were plentiful. During the recovery phase, red cell phospholipid concentrations approached normal levels again (0.726 picograms phospholipid per cell). The changes in cell cholesterol were much less dramatic than the changes in phospholipid. Concentrations remained relatively constant throughout the earlier phases of infection but rose progressively during the latter stages of the disease (Table 8).

Total phospholipid to cholesterol ratios were constant prior to initiation of infection and during the prepatent and recovery periods, with values of 2.8:1; 2.9:1; and 2.7:1 respectively. The patent period of
Table 8. Phospholipid and cholesterol composition of erythrocytes of normal and *Plasmodium gallinaceum* infected chickens.
Table 8.

<table>
<thead>
<tr>
<th>STAGE OF INFECTION</th>
<th>PHOSPHOLIPID PER RED CELL (×10^{-12} gr per rbc)</th>
<th>CHOLESTEROL PER RED CELL (×10^{-12} gr per rbc)</th>
<th>HEMATOLOGY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>psv</td>
</tr>
<tr>
<td>normal (preinfection)</td>
<td>0.550 ± 0.058 (12) -</td>
<td>0.196 ± 0.008 (21) -</td>
<td>28.0</td>
</tr>
<tr>
<td>prepateint</td>
<td>0.631 ± 0.045 (18) ns</td>
<td>0.218 ± 0.005 (9) s</td>
<td>27.0</td>
</tr>
<tr>
<td>early patent</td>
<td>0.791 ± 0.058 (15) s</td>
<td>0.196 ± 0.009 (12) ns</td>
<td>25.0</td>
</tr>
<tr>
<td>middle patent</td>
<td>1.166 ± 0.076 (11) s</td>
<td>0.225 ± 0.013 (12) s</td>
<td>20.0</td>
</tr>
<tr>
<td>late patent</td>
<td>1.198 ± 0.096 (12) s</td>
<td>0.248 ± 0.021 (10) s</td>
<td>17.0</td>
</tr>
<tr>
<td>recovery</td>
<td>0.726 ± 0.068 (6) ns</td>
<td>0.268 ± 0.011 (6) s</td>
<td>25.0</td>
</tr>
</tbody>
</table>

**= mean value ± standard error.

s= significantly different value from normal at 95% confidence level.

ns= not significantly different value from normal at 95% confidence level. Student's "t" test.

(n)= number sampled.
infection was characterized by high total phospholipid to cholesterol ratios.

The phospholipid concentration of a sample of red cells varied with the percentage of infected red cells and the percentage of basophilic erythrocytes in the sample. In chickens made anemic by phenylhydrazine injection, erythrocyte phospholipid and cholesterol concentrations increased in proportion to the percentage of basophilic cells in the sample (Table 9).

Individual malaria parasites contained about 0.68 picograms of phospholipid and 0.40 picograms of cholesterol. After adjustments were made in total red cell phospholipid concentration for the contributions of parasite phospholipids and phospholipids of young, immature cells, concentrations were in the normal range. Before infection there were 0.550 picograms of phospholipid per mature cell; at peak parasitemia after adjustment for parasite phospholipid there were 0.792 picograms; and during the late patent period of infection there were 0.559 picograms in each uninfected mature red cell. Cholesterol levels, in contrast, dropped progressively as the severity of infection increased. Adjusted red cell cholesterol values were 0.196 picograms per cell prior to infection; 0.089 picograms per cell at peak parasitemia; and finally 0.051 picograms per cell during the late patent phase of the infection.
Table 9. The effect upon red cell phospholipid and cholesterol concentrations of injecting chickens with phenylhydrazine.
### Table 9

<table>
<thead>
<tr>
<th>DAY POST-INJECTION</th>
<th>AMT. DRUG GIVEN PHENYLHYDRAZINE (IM)</th>
<th>PHOSPHOLIPID PER RED CELL (x10^{-12} gr per cell)</th>
<th>CHOLESTEROL PER RED CELL (x10^{-12} gr per cell)</th>
<th>HEMATOLOGY PCV %brbc</th>
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<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>$^{*}0.649 \pm 0.084$ (14)</td>
<td>$0.151 \pm 0.009$ (27)</td>
<td>27.0 0.0</td>
</tr>
<tr>
<td>1</td>
<td>5 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>$0.626 \pm 0.010$ (6)</td>
<td>$0.171 \pm 0.016$ (6)</td>
<td>24.4 5.1</td>
</tr>
<tr>
<td>3</td>
<td>10 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>$0.924 \pm 0.038$ (4)</td>
<td>$0.291 \pm 0.024$ (6)</td>
<td>26.8 23.0</td>
</tr>
<tr>
<td>5</td>
<td>10 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>nd</td>
<td>$0.292 \pm 0.010$ (6)</td>
<td>27.1 29.7</td>
</tr>
</tbody>
</table>

**= mean value + standard error.

nd= no determination made.

(n)= number sampled.
Plasma chemistry: Blood plasma chemistry was significantly altered as a result of *P. gallinaceum* infection (Table 10). Early in the infection plasma sodium levels fell and potassium concentrations increased. The plasma cation concentrations changed in a reciprocal relationship to the changes in intraerythrocytic sodium and potassium. During the late patent and recovery periods plasma potassium levels were below normal. The low plasma potassium levels were coincident to the elevated red cell potassium levels which occurred at this time. Plasma sodium concentrations were depressed throughout the entire course of infection and only returned to preinfection levels during the late recovery period. The mean pH of plasma remained constant prior to the onset of patent blood infection. During the middle and late patent phases of infection the pH of the blood plasma dropped. During the patent phase of infection a wider range of pH values than normal occurred. Concentration of plasma lipids was higher than normal. Phospholipids increased from preinfection value of 1.65 mg/ml early in the patent phase of infection and decreased thereafter until nearly normal values were reached in the recovery period. Plasma cholesterol levels were elevated throughout the infection but were highest during the late patent phase. Total plasma proteins increased during the patent phase of infection and remained elevated in the recovery period. UV
Table 10. Changes in plasma chemistry of chickens infected with *P. gallinaceum*.
Table 10.  

<table>
<thead>
<tr>
<th>DETERMINATION</th>
<th>SAMPLE PERIOD</th>
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<tr>
<td></td>
<td>PREINFECTION</td>
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<tr>
<td><strong>Hematology</strong></td>
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</tr>
<tr>
<td>hematology</td>
<td>30</td>
</tr>
<tr>
<td>$F$ irbe</td>
<td>0</td>
</tr>
<tr>
<td>$F$ brec</td>
<td>0</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
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<tr>
<td>sodium (mg/dl)</td>
<td>3.79±0.374</td>
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<tr>
<td>potassium (mg/dl)</td>
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<tr>
<td>chloride P (mg/dl)</td>
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<tr>
<td>phosphate (mg/dl)</td>
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<tr>
<td>pH</td>
<td>7.45±0.082</td>
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<tr>
<td>phospholipid (mg/dl)</td>
<td>1.65±0.086</td>
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<tr>
<td>cholesterol (mg/dl)</td>
<td>1.03±0.064</td>
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<tr>
<td>protein (total) (mg/dl)</td>
<td>4.32±1.43</td>
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<td>UV absorption</td>
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<tr>
<td>250nm</td>
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</tr>
<tr>
<td>260nm</td>
<td>2.31</td>
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<tr>
<td>280/260</td>
<td>1.52</td>
</tr>
<tr>
<td>Blood urea nitrogen (BUN) (mg/dl)</td>
<td>2.0</td>
</tr>
<tr>
<td>Blood sugar (mg/dl)</td>
<td>222.0</td>
</tr>
<tr>
<td>ACTase (U/1/min/dl)</td>
<td>0.0213</td>
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* = mean value ± SEM  
p = pooled plasma from six animals  
nd = no determination made
absorption at 280 nm by plasma collected during the patent phases of infection increased. There was a decrease in 280:260 UV absorption ratio during the late patent period of *P. gallinaceum* infection. Elevated urea nitrogen levels occurred during the late patent and recovery periods. Blood sugar decreased during the patent phase and then increased in the recovery period.

The most profound changes in blood plasma occurred during the middle and the late part of the patent period. Normal red cells incubated in plasma samples collected during these stages of infection were charged so that they physiologically resembled red cells from infected chickens in the acute phases of infection (Fig. 13). The late patent phase plasma samples, which caused an increase in osmotic fragility of normal red cells, had low pH's. When the pH of late patent phase plasma samples was adjusted to values close to the pH of preinfection plasma, the osmotic fragility inducing capacity was reduced but not entirely lost.

ATPase activity of normal erythrocytes incubated with patent phase plasma was also reduced. The ATPase activity of whole red cells treated with normal plasma was $1.14 \times 10^{-10}$ μM Pi/min/rbc. Red cells incubated with late patent phase plasma had an average activity
Figure 13. An osmogram of a multiple tube fragility test showing the increase in osmotic fragility of normal chicken erythrocytes treated with plasma from chickens in the middle and late patent phase of P. gallinaceum infection. Osmotic properties of red cells treated with blood plasma of chickens recovered from malaria or in the prepatent phase of infection are similar to those of red cells incubated with normal, preinfection plasma. Vertical line indicate range of values.
HEMOLYSIS

late patent

middle patent

normal

prepatent

recovery

% PHYSIOLOGICAL SALINE

% HEMOLYSIS
of 0.90 × 10^{-10} \text{ uM Pi/min/rbc. If the pH of these plasma samples was adjusted to the pH of preinfection phase plasma before the erythrocytes were incubated, then the inhibitory effects were reduced but still clearly evident.}

Trypsinization of erythrocytes from healthy chickens resulted in increased osmotic fragility and reduced total ATPase activity just as did incubation in patent phase plasma.

Both high (>50,000) and low (<50,000) molecular weight fractions of patent phase plasma had osmotic fragility inducing properties (Fig. 14). However, the patent phase low molecular weight filtrate was considerably more active in inducing fragility than the high molecular weight plasma fraction. Red cells incubated with low molecular weight fractions of pooled normal plasma were more osmotically resistant than red cells incubated in the reconstituted high molecular weight (>50,000) filtered normal plasma residue. The low molecular weight fraction of patent phase plasma had slightly lower pH's than normal plasma fractions and had elevated K^+ and decreased Na^+ concentrations. Incubation of normal red cells in patent phase low molecular weight plasma fractions (icp-low) reduced their ATPase activity 28%. Incubation of normal red cells with the equivalent, low molecular weight fractions of pooled normal plasma (ncp-low) also reduced red cell...
Figure 14. An osmogram of a multiple tube fragility test indicating the effects of pretreating red cells with fractions of normal and late patent phase plasma. Fractions of plasma were obtained by passing samples through membranes having molecular weight exclusion limits of 50,000, collecting the filtrate (low molecular weight fraction) and reconstituting the filtered plasma residue (high molecular weight fraction). Both the high and the low molecular weight fractions of patent phase plasma (icp) are more active in increasing osmotic fragility of normal red cells than are the control, pre-infection plasma fractions(ncp). Red cells incubated with low molecular weight patent phase plasma fractions are much more fragile than red cells treated with control, low molecular weight, normal plasma fractions. Vertical line indicate the range of values.
ATPase activity but to a lesser extent. The red cells incubated in late patent phase plasma and low molecular weight plasma ultrafiltrates, despite their greater fragility and decreased ATPase activity, did not have significantly shorter survival times than normal erythrocytes incubated in preinfection phase plasma when tested by the Chromium$^{51}$ technique (Fig. 15).
Figure 15. Effect of incubation in whole plasma and plasma ultrafiltrates on survival of Chromium$^{51}$ labeled erythrocytes. Figure 15A shows survival curves of erythrocytes incubated with plasma from healthy chickens and chickens in the late patent phase of infection. There is little difference between the two curves. Figure 15B shows the survival curves of erythrocytes treated with low molecular weight ultrafiltrates of normal and late patent phase plasma. Again the red cells incubated in fractions of plasma from acutely infected chickens were not more rapidly destroyed than those incubated in normal plasma filtrates.
DISCUSSION

A variety of interrelated changes occur in the morphology, physiology and biochemistry of red cells of chickens undergoing *P. gallinaceum* infection. The changes are induced by the infection and develop progressively as a consequence of it. Some of the changes are directly caused by the action of the parasites and some are indirect consequences. The magnitude of red cell alteration increases as a direct result of increasing parasitemia during early stages of the infection and is greatly influenced late in the infection by the basophilia which occurs as an indirect consequence of the infection.

Morphologic studies utilizing various light and electron microscopic techniques have indicated that early in the infection, when parasitemia is increasing, non-parasitized red cells have nearly normal morphology. Red cells containing small parasites also are almost normal in size and shape indicating that the merozoites produce little permanent morphologic damage by the act of penetration. The hypothesis (Ladda, 1969; Ladda et al., 1969) that the merozoite enters the erythrocyte by a phagocytic type process with the membrane pinching closed over the intracellular...
parasite is compatible with observations made here. The pinching off of the parasitic vacuole by the host cell membrane after entry would most probably be the cause of slit-like lesions (Fig. 4 C, D; arrows) which occur in red cell membranes near intracellular merozoites. Other investigators have reported what appear to be similar structures in EM thin-section. (Rudzinska and Vickerman, 1968; Trager, et al., 1966; Aikawa and Thompson, 1971). The suggestion that merozoite penetration results in little change in cell morphology is contrary to the observation of Arnold et al. (1969, 1971) who have suggested that penetration results in gross erythrocyte lesions and open holes in the cell surface through which parts of the parasite may protrude. Arnold et al. (1969, 1971) probably observed red cells which were caught by fixation while being penetrated by merozoites or erythrocytes containing large parasites which were undergoing lysis. Rinehart et al. (1971), used scanning electron microscopy in combination with a latex fixation procedure specific for erythrocyte membranes, to demonstrate that erythrocyte membranes over intracelluar parasites were distorted but still intact. This observation also supports the assertion that merozoite penetration leaves an intact erythrocyte membrane. Other workers concluded that in early phases of infection intracellular parasite growth is the major cause of gross erythrocyte alteration. Lewis and his
coworkers (1969) concluded from their ion-etching and scanning EM studies that red cell morphology becomes more abnormal as the parasite matures. These workers reported not only progressive distortion of gross morphology of the erythrocyte as the parasite developed but also modification of the limiting plasma membrane.

While the extent of morphologic alteration to the blood cells increases in proportion to the level of parasitemia during the early stages of patent parasitemia, late in the patent period of infection abnormal morphology is not limited to parasitized cells. A significant determining factor in the character of the blood cells during the late patent period of infection is the influx of new red blood cells produced under the stress of anemia. While the characteristics of these newly produced cells differ from the characteristics of cells in animals that have not undergone the stress of erythrocyte destruction their characteristics should not, except in the most critical way, be considered as part of the pathology of malaria.

The changes in morphology of erythrocytes from chickens with malaria just discussed parallel the changes in parameters of osmotic regulation reported in this study. Increases in red cell fragility are proportional to increases in parasitemia during the early phases of
infection just as during this period morphologic abnormality is largely limited to parasitized erythrocytes. Fogel et al. (1966) utilizing a recording fragilograph reported that increased osmotic fragility was characteristic of animals in the patent phase of Plasmodium gallinaceum, P. berghei, P. knowlesi, and P. falciparum infections and that a linear relationship existed between osmotic fragility and the percentage of parasitized red cells in blood samples from P. berghei infected hamsters. These workers also reported that nonparasitized erythrocytes separated from infected blood were more resistant to osmotic lysis than parasitized cells, but were less resistant than normal erythrocytes from healthy animals. -Shen and his collaborators (1946), in reporting the results of testing the osmotic and mechanical properties of Plasmodium knowlesi infected red cell populations, suggested that only those erythrocytes containing the larger and more fully developed parasites are abnormally fragile. This is in contrast to the observations made by various other investigators (Dannon and Gunders, 1962; Bahr, 1969) which indicate that both nonparasitized and parasitized erythrocytes are abnormally fragile. While these reports seem to be in conflict with one another, the conflict may be a result of failure of the workers to consider that not only is parasitemia changing during infection but the entire age composition of the erythrocyte population is changing also.
If one tests the osmotic fragility of erythrocytes in blood samples taken from *P. gallinaceum* infected chickens during the early patent period, one finds a direct relationship between the parasitemia and cell fragility. If one tests fragility of erythrocytes in blood samples from infected chickens collected during the late patent period of infection, a direct relationship between fragility and parasitemia is not observed. The stress of the anemia induced by the parasites induces production of defective reticulocytes which are fragile. Healthy chickens can be induced to produce similar fragile cells by stressing, by extensive bleeding or by injection of hemolytic drugs.

Even though nonparasitized erythrocytes collected during the latter part of the patent period are quite fragile, still they are more osmotically resistant to lysis than parasitized cells. This is the factor which is responsible for the overall decrease in fragility which follows peak parasitemia. The entry of some physiologically normal young erythrocytes into the blood probably contributes to the decrease in osmotic fragility which occurs during the late patent period. In the late recovery period when the cell population is made up of predominantly physiologically normal, young erythrocytes, the erythrocytes are significantly more resistant to osmotic lysis than erythrocytes in pre-infection samples.
Alterations in volume occur as changes in red cell fragility take place. Mean cell volume increase during the early patent period of infection is a result of volume change in parasitized erythrocytes. Volume changes in nonparasitized red cells do not contribute much to the total volume change. The extent of red cell volume change induced by parasitization depends on the parasite and host system. Mouse erythrocytes infected with *P. berghei* are reported to have mean cell volumes three times greater than normal. In contrast, heavily parasitized duck erythrocytes show little change in volume (Bahr, 1966). Volume increase may result from either increase in cellular water due to influx or increase in cytoplasmic substance. Weiss (1967) in our laboratory analyzed the buoyant densities of normal and *P. gallinaceum* infected erythrocyte samples by the copper sulfate, pycnometer and hydrometer float methods. By all three methods, parasitized cell samples had lower specific gravities (1.0404) than red cells from healthy chickens (1.0420). An influx of water into the cell was surely the cause of the volume increase which occurred in erythrocytes collected during the early patent period of infection. In the late patent period mean cell volumes were large due not only to the uptake of water by erythrocytes damaged by the infection but also due to the large numbers of large volume basophilic cytes within the blood samples.
The alterations in fragility and volume which occur during infection roughly parallel changes in intraerythrocytic cation balance. Changes occur in red cell sodium and potassium somewhat before changes are detected in fragility, volume or gross morphology. Even in the prepatent period cell sodium increases and potassium levels fall. The magnitude of intraerythrocytic cation imbalance increases as the infection progresses. Other investigators have noted similar changes in intracellular ion concentration in animals with a variety of experimental plasmodial infections eg: *P. berghesi*, *P. coatneyi*, *P. falciparum* (Dunn, 1969a); *P. knowlesi* (Overman, 1948; Dunn, 1969a); and the avian malarias, *P. gallinaceum* (Overman et al., 1950) and *P. lophurae* (Sherman and Tanigoshi, 1971). Kruszynski (1951) on the other hand reported increases in both cell sodium and potassium during *P. gallinaceum* infections. This result could have been obtained if the latter investigators' measurements were made on late patent phase blood samples in which erythrocyte potassium levels are high because of high basophilia. This explanation is suggested by observations on chickens stressed by phenylhydrazine injection which first undergo a hemolytic crisis and develop high cell sodium and low cell potassium levels and then when basophil production occurs develop high cell potassium levels. The cell sodium levels remain high because damaged erythrocytes with high sodium levels
are still present. The effect on cell cation concentration of the presence in the samples of damaged red cells and of cells of various physiological types complicates the interpretation of measurements of cation concentration in erythrocytes of the sample.

The abnormality of cation concentrations in red cells of infected animals may result from either an increased permeability of the membrane to these cations or to change in the activity of active transport mechanisms. Dunn (1969a,b) suggests that elevated erythrocyte sodium concentrations during plasmodial infection result from impaired active transport mechanisms. This is a reasonable suggestion since ATPase activity is depressed when parasitemias are high. Part of the suppressed ATPase activity must include the ouabain sensitive Na⁺/K⁺ ATPase transport system since total ATPase activity of uninfected erythrocytes is inhibited more by 10⁻⁴M ouabain than is the ATPase activity of infected cell populations. Incubating normal erythrocytes with plasma from acutely infected chickens depresses the total ATPase activity. This observation complements Dunn's (1969a) observation that there is a less active Na⁺ flux from normal erythrocytes treated with blood plasma of monkeys in the acute phase of P. knowlesi infection than from normal erythrocytes incubated in normal plasma. Cell alterations induced
in vitro by incubation in plasma are reversible while alterations resulting from parasitization are not. Red cells from *P. gallinaceum* infected chickens transfused into normal, healthy chickens are recognized as defective by healthy birds and removed at an accelerated rate (Schacter, 1969), whereas normal red cells which have increased osmotic fragility and decreased ATPase activity as a result of *in vitro* treatment have survival times *in vivo* similar to the survival times of normal cells treated with pre-infection plasma. It is probable that duration of exposure to the deleterious environment determines the reversibility of the damage.

A number of investigators have reported that red cells are altered antigenically during plasmodial infection and that these changes are similar to those induced in normal cells by *in vitro* treatment with trypsin (Kreier et al., 1966; Seed and Kreier, 1969; Gautum et al., 1970). It is therefore probable that red cells are altered by circulating proteolytic enzymes during the acute phases of the disease. Normal red cells which have been trypsinized, like red cells from animals with acute malaria have a reduced electrophoretic mobility (Seed, 1969), are osmotically fragile and a large portion of their total ATPase activity is lost. Trypsinization induces, as does infection, irreversible membrane damage as evidenced by shortened survival time after transfusion into healthy animals (Kreier, 1969). No one has looked
for proteolytic enzymes in plasma of malarious animals. If they occur there and act on erythrocytes their action would explain much of the pathology of malaria including ATPase activity reduction, fragility increase, and ion imbalance, as well as antigenic modification.

Various investigators have suggested that the red cell destruction in animals with malaria is due in part to a circulating toxin (Overman, 1948; Zuckerman, 1964; Bahr, 1969; Dunn, 1969a,b; Herman, 1969). Evidence for such a toxin is based on demonstration, by in vitro techniques, of the ability of infected plasma to alter normal red cell physiology and bring about increased osmotic fragility (Bahr, 1969; Herman, 1969) and change cation transport (Dunn, 1969a). The work presented here substantiates that plasma from acutely infected chickens affects normal erythrocyte function. However, the effect on erythrocytes of incubating them in plasma from animals with acute malaria was reduced by adjusting the pH of infected plasma to the pH of plasma prior to infection. Dunn (1969a) also reports that effects of infected plasma on cation transport are reduced greatly by adding certain metabolites and buffers to the plasma. These findings do not at all rule out the possibility that circulating toxins may contribute to cell pathology, but does confirm the belief that the physical state of the plasma during infection is a critical factor in production of cell pathology and premature erythrocyte senescence.
The increase in total plasma protein, particularly of the strongly acid γM (Abele et al., 1965) together with the reduction in pH which occurs during the acute phases of infection suppress the ionization of acidic groups on the erythrocyte and reduce its net negative charge and increase the viscosity of the cell and of its suspending fluids. The sludging and capillary blockage resulting in erythrocyte damage and phagocytosis commonly noted in malaria probably result from the high plasma viscosity and the low surface charge of the erythrocytes (Brown, 1933; Findlay and Brown, 1934; Krishnar et al., 1935; Mirady, 1969). A direct relationship between high plasma viscosity and short red cell life span has been experimentally demonstrated (Berlin, 1964). Murphy (1967) demonstrated that human red cells from healthy individuals and from patients with hereditary spherocytosis became more rigid and less filterable as the suspending fluids became more acid. The spherocytic cells were affected by pH change to a greater extent than normal erythrocytes. The pH values used by Murphy to demonstrate these effects were within the range of plasma pH values which occur in birds with *P. gallinaceum* infections. Murphy suggested that red cell destruction in people with hereditary spherocytosis was a result of increased blood viscosity and increased rigidity of spherocytes brought about by the low pH of the plasma. Similar pH affects probably occur in animals with malaria.
The reduction in plasma pH during the patent phases of malaria also has the adverse consequence of causing reduction in the oxygen binding capacity of hemoglobin (Rigdon and Rostorfer, 1946) and this in turn may be in part responsible for the anoxic state which develops during infection (Palecek et al., 1967).

The consequences of a reduction in the activity of the red cells cation pumps and the associated decrease in ATPase activity are far reaching. They include not only alteration in the intra and extracellular cation concentrations, water balance and osmotic fragility, but also alterations in the overall metabolic rate of the red cells because of effects on the intraerythrocytic ATP (Neerhout, 1968; Zarkowsky et al., 1968). It has been proposed that the intraerythrocytic level of ATP is a prime factor in determining the severity of malarial infection (Powell et al., 1966; Brewer and Coan, 1969). A positive correlation between red cell ATP levels prior to infection and the rate of parasite growth and multiplication has been reported (Brewer and Powell, 1965). One might postulate that a transitory increase in red cell ATP resulting from an inhibition of erythrocyte ATPase might be a critical factor in accelerating parasite buildup. A transitory increase in red cell ATP early in the patent period of P. berghei infection has been demonstrated (Brewer and Coan, 1969). The increase
occurs just as the decline in red cell ATPase, we have observed, begins. During the acute phases of *P. berghei* infection there is a significant decrease in red cell ATP (50%) far in excess of that which may be accounted for by parasite utilization (Brewer and Coan, 1969). The low ATP levels which occur as the disease progresses probably result in part from inactivation of ATP synthesizing systems resulting from feed back repression mechanisms linked with depressed erythrocyte ATPase activity.

In addition to partial control of cellular metabolism, the cation pump regulates the rate of turnover of red cell lipids (Jacob and Karnovsky, 1967). During the acute phase of *P. gallinaceum* infection when red cell ATPase activity is markedly depressed, the concentration of erythrocyte lipids is altered. Our studies indicated that in *P. gallinaceum* infected chickens the intracellular plasmodia account for nearly all the increase in phospholipids in the erythrocytes during the middle patent phase of infection. Similar results were reported for *P. berghei* infected erythrocytes (Lawrence and Cenedella, 1969; Cenedella et al., 1969) and *P. knowlesi* infected red cells (Rock et al., 1971). Erythrocyte phospholipid concentrations are slightly higher than normal during the early and middle patent phases of infection, but for all practical purposes the phospholipid concentration remains nearly constant throughout the course of infection. The dramatic increase in host
cell phospholipid reported by Rao et al. (1970) to occur in rats with *P. berghei* infection may have resulted from these authors insufficient adjustment for the contribution of parasite lipids and in the late patent phase of infection for the high phospholipid content of basophilic erythrocytes. While there is only minimal change from normal in host cell phospholipids during the patent period, if one excludes parasite and basophil phospholipid, there is a great decrease in erythrocyte cholesterol. The low erythrocyte cholesterol content might result from failure of plasma cholesterol to replace membrane cholesterol which is lost. The fact that high plasma cholesterol levels occur during the acute phase of infection suggests that such a process is occurring. That this is the case is also supported by the work of Cenedella et al. (1969) which indicates that normal erythrocytes incorporate labeled plasma cholesterol at a much greater rate than parasitized erythrocytes.

Murphy (1965) has shown that the shape and osmotic fragility of normal mammalian erythrocytes can be altered by changing red cell cholesterol concentrations or modifying cholesterol distribution in the membrane. Erythrocytes depleted of cholesterol are fragile. If the cholesterol is replaced the osmotic fragility decreases. In certain hepatic diseases such as Zieve's syndrome,
the patient's red cells have increased amounts of cholesterol and are often more resistant to osmotic stress than red cells of healthy individuals with normal lipid content (Westerman, 1968). As Neerhout (1968) points out, in a review of biochemical pathology in hematological disorders, in patients with hereditary spherocytosis there is a positive correlation between red cell lipid composition, altered cell permeability, activity of cation pumps and metabolic activity.

Pathology in animals with malaria is initiated by the plasmodial parasites which set in motion a chain of events not only in the physico-chemical condition of the red cells already present in the circulation but also initiate pathological and physiologically changes in other systems of the animal. Changes first occur primarily in parasitized cells. Later in the course of infection, when more parasites and parasite products and more products of cellular destruction are present, changes also occur in nonparasitized cells. These changes are similar to, but develop more rapidly than the alterations in normal red cells which occur with aging and senescence (Dannon, 1967). Modification of erythrocyte surfaces by toxic products which inactivate functional enzymes of active cation transport systems might be the initial process in a sequence of cellular events characterized by abnormal cation and water flux, lipid loss, and increased osmotic fragility.
Cell damage resulting in premature senescence of a large portion of the infected cell population initiates a response in the hemopoietic system which results in extensive change in the age distribution of the erythrocyte population and in the production of a population of crisis reticulocytes. The biochemical, physiological and morphological characteristics of the newly produced erythrocytes dominate the hematological picture from the late patent period through recovery. A population of these cells is not a normal equilibrium erythrocyte population in shape, size, membrane characteristics, or in general physiology. The characteristics of these newly produced cells are not the characteristics of cells made abnormal by the infection per se but are rather the characteristics of the cells contributing to the recuperative response of the host.

Hypersplenism and autoimmunity are the two physiological responses most frequently suggested as causes of pathology in malarious animals. Both are the direct result of the introduction into the circulation of large amounts of modified autologous, and heterologous antigens. Hypersplenism and autoimmunity develop as the parasites grow and destroy erythrocytes and regress rapidly when parasites actions end. The parasite is the cause of the disease, and all pathology is a consequence either direct or
indirect, of its actions. The change associated with recuperative mechanisms is not pathology. The reversibility of the course of the disease following specific chemotherapy should be enough to make us reject hypothesis in which consequences of parasitization, direct or indirect, are elevated to independently causal roles. The occasional occurrence of an anomalous situation, such as blackwater fever, following falciparum malaria, in which deranged physiological response becomes a prime cause of pathology should not confuse us as to the normal course of events and the role of the parasite in production of pathology.
SUMMARY

This study correlates changes in erythrocyte morphology during *Plasmodium gallinaceum* infection with physiological and biochemical alterations of the host cells. Gross morphologic abnormalities of red cells from infected chickens stem from loss in osmotic control, which is in part the result of inactivation of membrane-bound ATPase, cation imbalance and change in the concentration of erythrocyte lipids. Early in the patent period morphologic alterations are limited to parasitized erythrocytes, whereas physiologic abnormalities are not. Red cell ion imbalance and depressed ATPase activity precede morphologic change and parallel changes in the plasma pH, and in plasma lipid, cation and protein concentrations. The magnitude of abnormalities in erythrocytes, and the suspending plasma, increase as the infection progresses. These abnormalities in the red cells and in the plasma might be responsible for destruction of both parasitized and nonparasitized erythrocytes during infection. Late in the acute phase of infection and during the recovery period morphologic abnormalities occur in nonparasitized erythrocytes. Many of the abnormally shaped, nonparasitized cells are
basophiles. The influx into the diseased bird's circulation of many basophilic erythrocytes, which have elevated cholesterol and phospholipid content, high potassium concentration, increased ATPase activity and decreased osmotic fragility, greatly influences the physiologic as well as the morphologic characteristics of the red cell population and obscures the cellular pathology associated directly with the infectious process. The overall changes in cell physiology, biochemistry and morphology are induced by shifts in the age composition of the erythrocyte population during Plasmodium gallinaceum infection. Part of the change is due to premature senescence of cells already present in the population while some is due to replacement by young cells.
APPENDIX

Formula for solution

1. Ringer's solution, pH 7.3

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<td>4.6 mM</td>
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<tr>
<td>NaCl</td>
<td>123.0 mM</td>
<td>7.4240 &quot;</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.7 mM</td>
<td>0.1887 &quot;</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.9 mM</td>
<td>0.1596 &quot;</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.4 mM</td>
<td>0.7920 &quot;</td>
</tr>
<tr>
<td>HCl</td>
<td>20.4 mM</td>
<td>0.85 ml 6N/250 ml</td>
</tr>
<tr>
<td>THAM</td>
<td>21.8 mM</td>
<td>2.6378 gr/l</td>
</tr>
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