MOHAN, Ram, 1941-
STUDIES OF THE MORPHOLOGY AND SURVIVAL CHARACTERISTICS OF ERYTHROCYTES FROM MICE AND RATS WITH PLASMODIUM BERGHEI INFECTION.

The Ohio State University, Ph.D., 1971
Microbiology

University Microfilms, A XEROX Company, Ann Arbor, Michigan
STUDIES OF THE MORPHOLOGY AND SURVIVAL
CHARACTERISTICS OF ERYTHROCYTES FROM
MICE AND RATS WITH PLASMODIUM
BERGHEI INFECTION

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

By
Ram Mohan, B.V.Sc. and A.H., M.Sc.

The Ohio State University
1971

Approved by
Julius P. Kreier
Adviser
Department of Microbiology
ACKNOWLEDGMENTS

It is of ardent importance that an adviser set a fine example of close supervision, valued criticism, continuous encouragement and guidance for the successful completion of a graduate career for his students. To Dr. Julius P. Kreier I express my deep sense of gratitude and indebtedness for all of this.

I wish to thank Dr. Robert M. Pfister and Dr. Frank W. Chorpenning for their interest and help during my graduate training and for giving their valuable time for reading this manuscript.

I offer special thanks to Mr. Ivan Kapetanovic for his willing help with the electronmicroscopy and the preparation of photographic material.

Finally, I sincerely acknowledge the encouragement, assistance and sacrifices of my parents, and wife, Prabha, without which the attainment of this goal would have been impossible.

This work was supported in part by a United States Army Research Grant (DA-DA-17-68-C-8007) to Dr. J. P. Kreier.
VITA

August 15, 1941

Born - Gaya, India

1962

B.V.Sc. & A.H., The Bihar Veterinary College, Patna, India

1962 - 1966

Demonstrator, The Bihar Veterinary College, Patna, India

1967 - 1968

Research Assistant, Department of Poultry Science, The Ohio State University, Columbus, Ohio

1968

M.Sc., The Ohio State University, Columbus, Ohio

1968 - 1970

Research Associate, Department of Microbiology, The Ohio State University, Columbus, Ohio

1970 - 1971

Teaching Associate, Department of Microbiology, The Ohio State University, Columbus, Ohio

PUBLICATIONS


2. The Relationship Between Erythrocyte Morphology and Parasitization in Chickens, Rats and Mice Infected with Plasmodia. Submitted for Publication in Experimental Parasitology.
FIELDS OF STUDY

Major Field: Microbiology

Studies in General Microbiology - Professors Robert M. Pfister, James I. Frey and Bruno J. Kolodziej

Studies in Pathogenic Microbiology - Professors Matthew C. Dodd, Nancy Bigley, and Melvin S. Rheins

Studies in Bacterial Physiology - Professor Chester I. Randles

Studies in Immunology and Immunochemistry - Professor Frank W. Chorpenning

Studies in Virology - Professor David A. Wolff

Studies in Pathogenic Protozoology - Professor Julius P. Kreier
# 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>LIST OF ILLUSTRATIONS</td>
<td>ix</td>
</tr>
<tr>
<td>Chapter 1: INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1. Mechanisms of Erythrocyte Destruction during Malaria</td>
<td></td>
</tr>
<tr>
<td>A. Involvement of Hemolytic Substances</td>
<td>6</td>
</tr>
<tr>
<td>B. Increased Phagocytosis</td>
<td>7</td>
</tr>
<tr>
<td>C. Auto-immunization and Antibody mediated Erythrocyte Destruction</td>
<td>8</td>
</tr>
<tr>
<td>A. Erythrocyte Shape</td>
<td>12</td>
</tr>
<tr>
<td>B. Erythrocyte Surface</td>
<td>14</td>
</tr>
<tr>
<td>C. Erythrocyte Membrane</td>
<td>14</td>
</tr>
<tr>
<td>D. Red Cell Interior</td>
<td>15</td>
</tr>
<tr>
<td>3. Survival Studies of Normal Erythrocytes</td>
<td>16</td>
</tr>
<tr>
<td>4. Morphological Studies of Erythrocytes during Malarial Infection</td>
<td>20</td>
</tr>
<tr>
<td>5. Survival Studies of Erythrocytes during Malarial Infection</td>
<td>22</td>
</tr>
<tr>
<td>6. Physiological and Survival Studies of &quot;Stress&quot; Reticulocytes</td>
<td>25</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III. MATERIALS AND METHODS</td>
<td>31</td>
</tr>
<tr>
<td>1. General Techniques</td>
<td>31</td>
</tr>
<tr>
<td>A. Experimental Animals and Parasites</td>
<td>31</td>
</tr>
<tr>
<td>B. Hematological Techniques</td>
<td>31</td>
</tr>
<tr>
<td>2. Erythrocyte Morphological Studies</td>
<td>32</td>
</tr>
<tr>
<td>A. Phase and Nomarski Interference Microscope Studies</td>
<td>33</td>
</tr>
<tr>
<td>B. Light and Electron Microscope Studies of fixed Blood Cells</td>
<td>33</td>
</tr>
<tr>
<td>Blood Collection, Fixation and Washing</td>
<td>33</td>
</tr>
<tr>
<td>Light Microscope Studies by the Giemsa-staining and Carbon-shadowing Techniques</td>
<td>34</td>
</tr>
<tr>
<td>Combined Giemsa-staining light microscopy and Electron Microscope Carbon-replica Techniques</td>
<td>34</td>
</tr>
<tr>
<td>Combined Giemsa-staining light microscopy and Scanning Electron-Microscope Techniques</td>
<td>36</td>
</tr>
<tr>
<td>C. Morphological Studies of Rat Reticulocytes produced by Phenylhydrazine Hydrochloride Treatment</td>
<td>36</td>
</tr>
<tr>
<td>D. Morphological Studies of Rat Reticulocytes Produced by Repeated Cardiac Bleeding</td>
<td>36</td>
</tr>
<tr>
<td>3. Erythrocyte Survival Studies</td>
<td>37</td>
</tr>
<tr>
<td>A. Experimental Design</td>
<td>37</td>
</tr>
<tr>
<td>B. Experimental Techniques</td>
<td>38</td>
</tr>
<tr>
<td>Labeling of Erythrocytes with Chromium</td>
<td>38</td>
</tr>
<tr>
<td>Sampling of Blood</td>
<td>39</td>
</tr>
<tr>
<td>Counting and Data Processing</td>
<td>39</td>
</tr>
<tr>
<td>IV. RESULTS</td>
<td>41</td>
</tr>
<tr>
<td>1. General Course of <em>P. berghei</em> infection in Mice and Rats</td>
<td>41</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Light microscope observations of Mouse and Rat erythrocytes during P. berghei infection</td>
<td>44</td>
</tr>
<tr>
<td>A. The appearance of Giemsa stained erythrocytes</td>
<td>44</td>
</tr>
<tr>
<td>B. The appearance of fresh blood cells by Phase Contrast and Nomarski Interference Microscopy</td>
<td>52</td>
</tr>
<tr>
<td>C. The appearance of Giemsa stained and Carbon Shadowed gluteraldehyde fixed erythrocytes</td>
<td>62</td>
</tr>
<tr>
<td>3. Appearance of Mouse erythrocytes during P. berghei infection</td>
<td>67</td>
</tr>
<tr>
<td>A. The combined technique using Giemsa staining and Light Microscopy followed by Electron Microscopy of carbon replicas of the previously examined erythrocytes</td>
<td>67</td>
</tr>
<tr>
<td>B. The combined technique using Giemsa staining and Light Microscopy followed by Scanning Electron Microscopy of the same erythrocytes</td>
<td>79</td>
</tr>
<tr>
<td>4. Appearance of erythrocytes from Rats with P. berghei infections</td>
<td>82</td>
</tr>
<tr>
<td>A. The combined technique of Giemsa staining and Light Microscopy followed by Electron Microscopy of carbon replicas</td>
<td>82</td>
</tr>
<tr>
<td>B. The combined technique of Giemsa staining and Light Microscopy followed by Scanning Electron Microscopy</td>
<td>87</td>
</tr>
<tr>
<td>5. Morphological observations of Rat reticulocytes produced following Phenylhydrazine treatment</td>
<td>88</td>
</tr>
<tr>
<td>6. Morphological characterization of Rat reticulocytes produced following repeated Cardiac bleeding</td>
<td>94</td>
</tr>
<tr>
<td>7. Survival of Rat Erythrocytes labeled with Cr\textsuperscript{51} and transfused into Normal Animals</td>
<td>94</td>
</tr>
<tr>
<td>A. Normal Erythrocytes</td>
<td>94</td>
</tr>
<tr>
<td>B. Infected Erythrocytes (50% parasitemia)</td>
<td>99</td>
</tr>
<tr>
<td>C. Basophilic Erythrocytes</td>
<td>102</td>
</tr>
<tr>
<td>D. Post Infection Mature Erythrocytes</td>
<td>107</td>
</tr>
<tr>
<td>V. DISCUSSION</td>
<td>110</td>
</tr>
<tr>
<td>Chapter</td>
<td>Page</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>VI. SUMMARY AND CONCLUSIONS</td>
<td>123</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>126</td>
</tr>
<tr>
<td>Formulas for Solutions</td>
<td>127</td>
</tr>
<tr>
<td>Tables</td>
<td>128</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>143</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Graph showing the usual course of <em>P. berghei</em> infection in adult mice</td>
<td>43</td>
</tr>
<tr>
<td>2</td>
<td>Graph showing the usual course of <em>P. berghei</em> infection in adult rats</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>Photomicrographs of Giemsa stained erythrocytes from mice in various stages of malarial infection</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td>Photomicrographs of Giemsa stained erythrocytes from rats in various stages of malarial infection and recovery</td>
<td>51</td>
</tr>
<tr>
<td>5</td>
<td>Photomicrographs by the phase contrast technique of erythrocytes from mice in various stages of malarial infection</td>
<td>54</td>
</tr>
<tr>
<td>6</td>
<td>Nomarski interference photomicrographs of erythrocytes from mice in various stages of malarial infection</td>
<td>56</td>
</tr>
<tr>
<td>7</td>
<td>Photomicrographs by phase contrast of erythrocytes from rats in various stages of malarial infection</td>
<td>59</td>
</tr>
<tr>
<td>8</td>
<td>Photomicrographs by the Nomarski interference technique of rat erythrocytes showing various effects of parasitization.</td>
<td>61</td>
</tr>
<tr>
<td>9</td>
<td>Photomicrographs of carbon shadowed Giemsa stained erythrocytes from mice in various stages of malarial infection</td>
<td>64</td>
</tr>
<tr>
<td>10</td>
<td>Photomicrographs of carbon shadowed Giemsa stained erythrocytes from rats in various stages of malarial infection</td>
<td>66</td>
</tr>
<tr>
<td>11</td>
<td>Photomicrographs of Giemsa stained normal mouse erythrocytes and electron micrographs of the same erythrocytes</td>
<td>69</td>
</tr>
<tr>
<td>12</td>
<td>Photomicrographs of Giemsa stained erythrocytes of a mouse infected with malaria (20% parasitemia) and electron micrographs of the same erythrocytes</td>
<td>71</td>
</tr>
<tr>
<td>13</td>
<td>Photomicrographs of Giemsa stained erythrocytes of a mouse infected with malaria (50% parasitemia) and electronmicrographs of the same erythrocytes</td>
<td>73</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>14.</td>
<td>Photomicrographs of Giemsa stained erythrocytes of a mouse infected with malaria (70% parasitemia) and electronmicrographs of the same erythrocytes</td>
<td>75</td>
</tr>
<tr>
<td>15.</td>
<td>Photomicrographs of Giemsa stained erythrocytes from mice in various stages of malarial infection and electron micrographs of the same erythrocytes</td>
<td>78</td>
</tr>
<tr>
<td>16.</td>
<td>Photomicrographs of normal and malaria infected mouse erythrocytes and scanning electron micrographs of the same erythrocytes</td>
<td>81</td>
</tr>
<tr>
<td>17.</td>
<td>Electron micrographs of erythrocytes from rats in various stages of malarial infection and recovery with photomicrographs of the same erythrocytes as inserts</td>
<td>84</td>
</tr>
<tr>
<td>18.</td>
<td>Electron micrographs of erythrocytes from rats in various stages of malarial infection and recovery with photomicrographs of the same erythrocytes as inserts</td>
<td>86</td>
</tr>
<tr>
<td>19.</td>
<td>Photomicrographs of erythrocytes from rats, before infection, during patent parasitemia and post infection and scanning electron micrographs of the same erythrocytes</td>
<td>90</td>
</tr>
<tr>
<td>20.</td>
<td>Electron micrographs of erythrocytes from a rat injected with phenylhydrazine</td>
<td>93</td>
</tr>
<tr>
<td>21.</td>
<td>Electron micrograph of erythrocytes of a rat bled repeatedly from the heart</td>
<td>96</td>
</tr>
<tr>
<td>22.</td>
<td>Survival curve of normal erythrocytes transfused from a normal inbred rat to compatible inbred non-infected rats</td>
<td>98</td>
</tr>
<tr>
<td>23.</td>
<td>Survival curve of erythrocytes transfused from <em>P. berghei</em> infected rats (50% parasitemia) to compatible drug treated non-infected rats</td>
<td>101</td>
</tr>
<tr>
<td>24.</td>
<td>Survival curve of basophilic erythrocytes (80% basophilia) transfused from a rat recovering from <em>P. berghei</em> infection to compatible drug treated non-infected rats</td>
<td>104</td>
</tr>
<tr>
<td>25.</td>
<td>Survival curves of normal and post infection basophilic erythrocytes</td>
<td>106</td>
</tr>
<tr>
<td>26.</td>
<td>Survival curve of mature erythrocytes present in a rat 15 days after the end of patent parasitemia</td>
<td>109</td>
</tr>
</tbody>
</table>
INTRODUCTION

Malaria, once a widespread disease and greatest single killer of the human race, has been known to exist in the world since antiquity. In spite of more than a decade of operation of a world wide malaria eradication program, malaria still has a great impact on the health of populations of many nations. The dramatic outbreak of the disease in Ceylon (Gochenour, 1969)*, where malaria had been thought to be eradicated, indicates the difficulties in the way of completion of eradication. In addition, after taking into account the increasing resistance of the anopheles to pesticides, and of plasmodia to drugs, it appears fair to state that malaria will continue to be a major problem for the foreseeable future. A better knowledge of the parasite and its interactions with various hosts may help in finding a solution to the malaria problem.

The importance of rodent malaria mainly lies in its being an excellent research tool for the malariologist. Malaria research was handicapped for a long time by lack of a suitable animal model. Since the discovery of *Plasmodium berghei* (Vincke, 1948), rodent malaria has been extensively used for screening various anti-malarial drugs, and for learning about human malaria.

As the malaria parasite mainly infects and destroys red blood cells, studies of blood changes and anemia have always been a subject of great importance. It has been observed by many workers (Clark et al., 1949;
that during malarial infection erythrocyte destruction and anemia may exceed the level which can be expected due to the destruction of parasitized cells alone. Release of hemolytic substances by the parasites or parasitized cells, antigenic modification of blood cells, and stimulation of autoimmune responses have been postulated to explain the destruction of erythrocyte in individuals with malaria. Most of these hypotheses suggest that both parasitized as well as non-parasitized red blood cells are modified and destroyed during the infection. The extensive phagocytosis of non-parasitized cells, observed by several workers during studies of animals and man with malarial infection suggests that there is some kind of modification even of non-parasitized erythrocytes.

In spite of hypotheses suggesting that there is modification or damage to both parasitized and non-parasitized cells, there have been few attempts to evaluate the morphology of the erythrocytes during malarial infection. Most of the electron microscope studies of the erythrocytes from animals with malaria have been done by the transmission electron microscope technique, which gives only limited information about the gross morphological changes in blood cells. Arnold et al. (1969) reported scanning electron microscope observation of malaria infected red blood cells, and indicated the presence of big holes in both parasitized and non-parasitized blood cells. His report was immediately challenged (Sprinz, 1969; Trager, 1969), and is still a subject of controversy.

This study was initiated to examine (by various light and electron microscope techniques) the morphology of both infected and non-infected
erythrocytes in animals with plasmodial infection, and to correlate the morphological observations with the pathology of the disease.
REVIEW OF LITERATURE

1. Mechanisms of Erythrocyte Destruction during Malaria

Malaria is a disease caused by parasites of the genus *Plasmodium* which belongs to the sub-order Haemosporidiiidae of the class Sporozoa. Schizogony and gametogony of the malarial parasites occurs in the blood cells of men, animals, and birds, and the disease is characterized by destruction of erythrocytes and production of anemia. One of the apparent causes of erythrocyte destruction is the growth in the erythrocyte of the parasite itself. During the erythrocytic stage of the infection the merozoites enter the red blood cells, mature and multiply intracellularly using the hemoglobin of the cells, and finally a new crop of merozoites comes out of the cells causing cell rupture. Kitchen (1949) indicated that cell rupture during the merozoite release was the main cause of erythrocytes destruction and malarial anemia.

However, it has been observed (Taliaferro, 1936) that large numbers of infected erythrocytes are destroyed by phagocytosis before the parasites mature. This is probably a result of an attempt by the normal defense mechanism of the host to get rid of infection. But, when increased phagocytic activity occurs a great number of uninfected erythrocytes are also phagocytized. Phagocytosis of uninfected erythrocytes continues for several days beyond the stage of removal of most of the infected erythrocytes.
Zuckerman (1957) reported observations on the interrelationships among parasitemia, erythrocyte destruction and the subsequent replacement of erythrocytes in rats infected with *Plasmodium berghei*. She noticed a discrepancy between the extent of erythrocyte destruction and degree of parasitemia, and indicated that this was due to cell destruction by methods in addition to rupture of erythrocytes during merozoite release. Later she examined plasmodial infection in various hosts and concluded that anemia produced in all cases was much greater than could be accounted for by parasitemia alone (Zuckerman, 1963). Observations similar to those of Zuckerman were also reported by several other workers (Clark et al., 1949; McGhee, 1964; Sloan et al., 1965).

The phenomenon of erythrocyte destruction by causes other than rupture of cells during release of parasites is not unique to malaria alone. Even in the case of certain forms of trypnosomiasis anemia occurs, although the parasite never enters erythrocytes. Several hypotheses have been postulated to explain destruction of non-parasitized erythrocytes in malaria.

Important among them are:

A. Involvement of hemolytic substances produced by the parasite or released due to rupture of cells.

B. Increased phagocytosis due to enlarged spleen or increased activity of individual macrophages.

C. Autoimmunization and antibody mediated red cell destruction.

A consideration of these hypotheses appears necessary to understand the basis of their recommendation, and their significance in the overall process of erythrocyte destruction in malaria.
A. Involvement of Hemolytic Substances

The occurrence of massive erythrocyte destruction in black water fever patients during or following *Plasmodium falciparum* infection in humans can not be explained by erythrocyte destruction by direct parasite action. Most commonly this pathological condition has been considered to result from the action of a hemolytic factor, possibly an autoantibody.

After maturation of a schizont inside an erythrocyte, the erythrocyte ruptures and as a result large amounts of parasitic excretory products, cell debris and enzymes are released into the general circulation. It is possible that some of these materials might behave like toxins and cause lysis of uninfected erythrocytes. An unsaturated fatty acid was suggested as a hemolytic factor in malaria by Laser (1950). He observed a 25 to 75 fold more hemolytic factor in the plasma of animals with high parasitemia than in those with low levels of parasitemia; and the amount present varied in direct proportion to the degree and duration of the infection. He, therefore, believed that the malarial parasites inside erythrocytes produce a hemolytic substance, which destroys the erythrocytes when its concentration is high, and which is then released into the plasma. In addition, another parasite metabolite, malaria pigment, is hemolytic in concentrations as low as 1 part in 50,000 and in concentrations as low as 1 part in 200,000 strongly potentiates the effect of the hemolytic substance.

The observations of many other workers also suggest the existence of circulating toxins in animals with malarial infection. Riley and Maegraith (1961) have shown that the serum of monkeys infected with *P.*
knowlesi contains a factor which inhibits the respiration and uncouples oxidative phosphorylation of hepatic mitochondria from either mice or monkeys.

E. Increased Phagocytosis

One of the normal functions of the spleen is to remove from the general circulation by phagocytosis abnormal and aged blood cells. During malarial infection the size and phagocytic capacity of the spleen greatly increases. There are several reports in the literature stating that in addition to the parasitized blood cells, non-parasitized erythrocytes are also phagocytised (Taliaferro and Mulligan, 1937; Zuckerman, 1945; Taliaferro and Taliaferro, 1955).

Motulsky et al. (1958) in a discussion of hypersplenic anemia suggested that perhaps normal erythrocytes are damaged during retention in the enlarged spleen. Such damage, if it occurs, would be increased during repeated circulation through the large spleen, then finally he postulated that the damaged erythrocytes are removed from the circulation by phagocytosis.

Zuckerman (1966) observed that as P. berghei infection progressed there was a more than 250 fold increase in the amount of phagocytosis of unparasitized blood cells by spleen macrophages. Based on these observations and on observations of autoantibodies in rats with malaria, Zuckerman (1960) suggested that immune sensitization of both infected and uninfected erythrocytes may occur in P. berghei infected rats.

George et al. (1966) on the other hand proposed that the anemia in rats infected with P. berghei is caused by hypersplenism without involvement of autoimmunity. Their belief was based on the fact that they
found neither evidence of antierythrocytic antibody by direct or indirect Coombs tests nor any decrease in erythrocyte survival times for erythrocytes from infected donor rats transfused into normal rats.

Zuckerman et al. (1969) studied the significance of hypersplenism in production of anemia in rats with sterile splenomegaly produced as a result of injection of methyl cellulose. By this technique it was only possible to produce a mild splenomegaly which was similar in extent to the splenomegaly seen in the early and latent stages of infection. The anemia produced in rats with mild splenomegalies was similar in extent to the anemia produced during the early and latent stages of infection. As it was not possible to induce sterile splenomegaly comparable to that which occurs during the crisis period of infection, she reserved her comments on the role of hypersplenism as the sole cause of destruction of non-parasitized erythrocytes in animals with malarial anemia. In addition, she suggested that demonstration of an elutable hemagglutinin and evidence of increased phagocytosis by individual splenic phagocytes in malarial rats raises the question of involvement of other anemia producing factors in addition to hypersplenism.

C. Auto-immunization and Antibody mediated Erythrocyte Destruction

It is well known that erythrocytes may be destroyed following combination of antibody with foreign antigens absorbed on the surface of the erythrocyte. When parasitized blood cells rupture large amounts of parasite antigens which may be parasite excretory products, and parasite and host cell enzymes, etc. are released into the general circulation. Eaton (1939) found soluble antigen in the serum of monkeys infected with *P. knowlesi*, and the amounts of antigen correlated with
the degree of parasitemia. It has been postulated that uninfected blood
cells might become coated with parasitic antigen and be removed from
the circulation after sensitization with antiparasitic antibody (Zuckerman, 1964). In addition it is also possible that due to the action of
parasite excretory products or enzymes, the normal blood cell antigens
may become modified and thus stimulate the production of autoantibody.

The alteration of the normal erythrocyte surface by enzyme action
may lead to exposure of new antigenic sites was shown by Dodd et al.
(1953). They reported that injection of trypsinized erythrocytes into
rabbits stimulated the production of antitryptsinized erythrocytic anti­
body. This antibody agglutinated trypsinized human red blood cells as
well as blood cells from patients suffering from acquired hemolytic
anemia.

Kreier et al. (1966) demonstrated an agglutinin for trypsinized
autologous and homologus erythrocytes in the sera of rats infected with
P. berghei. They also eluted a similar agglutinin from erythrocytes of
P. berghei infected rats by heating at 37°C for 45 minutes. These
agglutinins produced similar electrophoretic patterns and showed similar
susceptibility to mercaptoethanol treatment. Kreier et al. further
explained that, if the antibody to the modified erythrocytes sensitized
them to phagocytosis, it is possible that this action could coincidentally
aid the animal in clearing its circulation of intracellular parasites
which would otherwise be protected from the host defense mechanisms by
their intracellular location.

Fogel et al. (1966) observed that the complement activity of sera
from monkeys, hamsters and chickens with terminal malaria infections
declines in direct relation to the severity of the disease. This, they indicated, was probably due to formation of antigen, antibody and complement complexes in the circulation. Such complexes might be adsorbed on the surface of normal erythrocytes and either cause their intravascular hemolysis or opsonize them so that they are phagocytized.

There are several reports of in vivo globulin coating of erythrocytes in hemolytic anemias. Dacie (1962) investigated 92 patients suffering from auto-immune hemolytic anemia and obtained positive antoglobulin reactions in each case.

Zuckerman (1960) reasoned that a similar phenomenon could be responsible for destruction of non-parasitized erythrocytes in animals with malaria. She thereafter studied animals with malaria by a modified Coombs test and demonstrated antibody coating of cells at, and after the crisis of infection with P. berghei and P. vinckei (Zuckerman and Spira, 1961). However, the results of these tests were questioned when they (Zuckerman and Spira, 1961) also obtained positive Coombs reactions in rats with nonparasitic anemias, notably those induced by serial cardiac bleeding or by the administration of phenylhydrazine hydrochloride. The positive Coombs tests, they explained, were due to coating of a beta-globulin called transferrin on the reticulocyte surfaces and were not a result of autoantibody.

George et al. (1966) further investigated antibody coating of erythrocytes of animals with malaria by Coombs tests using antisera specific for the Ig G (7s) fraction of gamma globulin. They did not obtain any evidence of globulin coating on erythrocytes of rats with malaria. On the other hand, Gautam et al. (1970), by using anti Ig M
antibodies and a sensitive radio-immuno-assay technique, obtained evidence of globulin coating in addition to transferrin on erythrocytes of chickens at least during certain stages of *P. gallinaceum* infection.

In addition to the factors discussed earlier, some additional physiological changes in erythrocytes occur which might play some role in the destruction of erythrocytes of animals with malaria. Maegraith (1954) indicated that the electrical charge of both infected and uninfected erythrocytes is reduced during malarial infection. Recently similar reduction in charge was noted by Nirady (1969) who worked with erythrocytes from chicken with *Plasmodium gallinaceum* infection. She observed a decrease of electrophoretic mobility of both infected and uninfected blood cells especially at the time antibody production occurred. She also noticed that both infected and normal erythrocytes showed decreased electrophoretic mobility after suspension in infected serum. Similarly, in normal serum the infected and uninfected cells also showed decreases in electrophoretic mobility, but those decreases were less than those observed on suspension in infected serum. These observations indicate that some factor associated with the serum of infected animals, possibly antibody, might be responsible for the decrease in the electrophoretic mobility of the cells.

The occurrence of increased osmotic fragility of erythrocytes during malarial infection has been known for a long time (Geiman et al., 1946). Shen et al. (1946) observed that the erythrocytes carrying shizonts were most fragile and they speculated that probably only the infected cells are affected by the parasite growth and become more fragile. But some recent work does not support the above hypothesis.
Danon and Gunders (1962), and Fogel et al. (1966) reported an increase in the osmotic fragility of both infected and noninfected cells. Bahr (1969) indicated that some humoral factor might be responsible for the increase in the osmotic fragility of even noninfected cells. This hypothesis was based on the fact that he observed an increase in the fragility of normal cells following incubation for half an hour in the plasma of malaria infected animals, but no such effect after incubation of infected cells in normal plasma. It is possible that the increase in the osmotic fragility of normal erythrocytes which occurred after incubation of the cells in the plasma of infected animals might have been due to the action of hemolytic substances present in the plasma of malaria infected animals or adsorption on the surface of the normal erythrocytes of some materials, such as antigen-antibody complexes from the plasma.

Dunn (1969) reported alteration in sodium and potassium transport of blood cell membranes during malarial infection in monkeys. He presented evidence that there was elevated red blood cell sodium as a consequence of diminished sodium outflux and increased sodium influx in both parasitized and non-parasitized blood cells. He noticed a slow and progressive deterioration of membrane function over a period of days probably leading to increase in the fragility and eventually to hemolysis of those cells.


A. Erythrocyte Shape

The discoidal, biconcave shape of a typical mammalian red blood cell has been familiar to most people for a long time. In spite of a
great deal of work on the part of biochemists, physiologists, and biophysicists a satisfactory explanation for the biconcave shape is still not available. It has been suggested that the biconcave shape is most suitable for quick diffusion of materials and permits moderate volume change without stretching the cell membrane.

According to Baker (1967), one of the earliest ideas on the blood cell biconcavity was put forward by Howell in 1891, who ascribed the initial biconcavity to the expulsion of the nucleus, which would cause a sagging in of the membrane. A concept of an internal protein framework was also considered as an explanation, but recent thin section electron microscope work (Baker, 1967) shows no evidence for the existence of such a framework.

Murphy (1965) who used tritiated cholesterol in an autoradiography technique showed that cholesterol is concentrated around the periphery of the biconcave disc of human red cells. Murphy proposed that the uneven distribution of cholesterol over the cell surface caused inequalities in surface tension because of uneven exposure of hydrophobic groups, and that these differences in surface tension played roles in determining cell shape. It is probable that differences in the membrane structure and composition other than cholesterol distribution might also play some part in determining the cell shape.

The use of various electron microscope techniques has significantly helped our understanding of ultra-structure of the red cell. Before the advent of thin sectioning the examination of red cells was restricted to the outer surface of intact or hemolysed cells, mostly by replication techniques.
B. Erythrocyte Surface

Early workers like Angulo (1949), and Bessis (1956) etc. reported the presence of craters, and crystaline and myelin structures on the surface of red blood cells. It is now recognized that many of these forms were either derived from the plasma or were gross artifacts.

Hillier and Hoffman (1953) carried out careful examinations of human red cell ghosts by use of chromium shadow techniques. They demonstrated the existence of plaques 30 Å thick and 100 to 500 Å in diameter arranged apparently at random over the surface. As the plaques were removed from the surface by ether, but remained intact when free, they concluded that the plaques are bound to the membrane by lipid.

Hoffman (1956) also carried out a comparative study of the ultrastructure of blood cell ghosts of various mammalian species. He not only observed differences in the surface texture of blood cells of different species of animals, but the blood cells of the same animal collected on the same day.

Glaeser and coworkers (1966, 1967) examined the surface structure of nucleated and non-nucleated red blood cells fixed with osmium, metal shadowed and carbon replicated. They reported pebbly or granular structures (400 to 500 Å in diameter) randomly arranged on the surface of both nucleated and non-nucleated erythrocytes.

C. Erythrocyte Membrane

The use of thin sectioning techniques in electron microscopy has helped us to obtain a better knowledge of blood cell membrane structure. Early electron micrographs of red cells usually showed a single dense line at the periphery. Recent improvements in embedding, cutting and
staining techniques have resulted in the resolution of the single line
and reveal the plasma membrane to be three layered complex, made up of
two dark-lines, each 25 Å thick separated by a less dense interspace 20
Å thick. This structure is not unique to the red cell membrane, rather
it is found in many other cell types as well, and has been given the
name "unit membrane" by Robertson (1960).

The unit membrane of Robertson tends to confirm the Davidson-
Danielli model of the membrane as a bimolecular leaflet of lipid with
protein at both faces. Many workers have not wished to accept the
concept of the unit membrane because there are wide functional varia-
tions in cell membranes. Solomon (1960) suggested that it is possible
that only a small percentage of the total surface area might have
specialized function and structure. Whittman (1964) has proposed a
model of the red cell membrane which attempts to incorporate most of
the available physical and chemical data. The plaques of Hillier and
Hoffman are aligned underneath an outer glycoprotein layer, while a
bimolecular lipid layer, lined on both surfaces by Ca++ ions, accounts
for the unit membrane. A layer of protein tied to the underside of the
bimolecular leaflet by Ca++ ions is suggested to be in direct contact
with the hemoglobin.

D. Red Cell Interior

The thin section electron microscope technique has been used for
some time to study the interior of the blood cell, recently the freeze-
etching technique has been introduced. The latter technique appears
promising because it does not involve any chemical fixation.
Most of the thin section studies indicate that the blood cell interior has an amorphous nature. Haggis (1961) indicated that with the help of freeze-etching it is possible to recognize the four molecular subunits of individual hemoglobin molecules.

Similar results were obtained by Weinstein and Bullivant (1966) in a study of freeze-etching of red cells. They observed a fairly uniform granularity (granules approximately 100 Å in diameter) inside the red cells and suggested that the granules were replicas of individual hemoglobin molecules.

3. Survival Studies of Normal Erythrocytes

Survival studies on erythrocytes have been of great interest because of their importance in studies of various hemolytic diseases. Before the introduction of isotope labeling methods, the Ashby technique was the only method available for studying erythrocyte survival. This technique was useful mainly in man. The Ashby technique involved transfusing group "O" blood into a group "A" or "B" individual and noting the rate of "O" cell removal by a differential agglutination technique using iso-antibody. Due to the requirement for iso-antibody the technique had limitations for use in study of erythrocyte survival of animals and could not be used for study of autologously transfused blood. The introduction of various isotopic labeling techniques has literally brought a revolution in erythrocyte survival studies of man and various animals. These techniques can be roughly divided into two classes, those in which the radioactive nuclides label cells of a limited age group as by use of N⁺¹⁵, C¹⁴, Fe⁵⁵ and Fe⁵⁹ etc., and those in which
all the cells are labeled regardless of age as by use of Cr^{51} or P^{32} as DFP^{32}.

Erythrocyte Labeling with Cr^{51}

The usefulness of radioactive sodium chromate (Na Cr^{51} O_4) as a tracer substance for the measurement of blood volume was first reported by Gray and Sterling (1950). These investigators found in labeling red cells with radioactive sodium chromate, that 97% of the radioactive nuclide was associated with the hemoglobin, and of this 97%, 78% was recovered from the globin fraction. Due to faster disappearance of Cr^{51} from the blood than was expected to occur on the basis of the rate of erythrocyte destruction, they expressed doubt of the usefulness of the technique for erythrocyte survival studies.

Ebaugh et al. (1953) reported that slow elution of Cr^{51} from the labeled cell was the main cause of the fast disappearance of Cr^{51} from the blood. They indicated that Cr^{51} is eluted slowly at an exponential rate, and is not reutilized to tag new cells. They indicated that Cr^{51} can be utilized for erythrocyte survival studies if one makes a correction for the Cr^{51} elution.

Necheles et al. (1953) studied elution of chromium from human blood cells, and indicated that the elution takes place at a constant rate of approximately one percent of the remaining radioactivity per day. After using 1% elution rate as correction factor, they obtained an erythrocyte survival curve comparable to that obtained by other techniques.

Mollison and Veall (1955) reported that the removal of chromium label from the circulation occurs in at least two phases. They observed
a faster removal of chromium in the first 24 hour period after labeling than in subsequent periods.

Kleine and Heimpel (1965) using simultaneous labeling of human erythrocytes with Cr\(^{51}\) and P\(^{32}\), observed that rapid early loss of Cr\(^{51}\) activity was due to a rapid elution of a portion of the label rather than destruction of the erythrocytes.

Danon et al. (1966) suggested that as leucocytes and platelets have a much greater affinity for chromium than erythrocytes, the greater loss of chromium during the initial 24 hour period after transfusion might be partially due to loss of these short lived cells.

Necheles et al. (1953) studied the effect of radioactive sodium chromate on red blood cells. They indicated that at a dose of 200 μc per 100 ml blood there was not any injury to normal blood cells. However increasing the dose fifteen fold increased cell fragility.

Donohue et al. (1955) studied the survival of blood cells in rabbits and humans using various concentrations of chromium. They could not observe any adverse effect on cells below a dose of 25 μc of chromium per ml of blood, but a slight shortening of the cell life span was noticed at 50 μc chromium per ml blood, and 100 μc of chromium per ml of blood causes a marked reduction in the erythrocyte survival.

Hughes-Jones and Mollison (1956) also studied the effect of high concentrations of sodium chromate on erythrocytes and indicated that a dose 30 to 40 μc per ml of blood caused reduction in the cell survival together with a sudden disappearance of labeled cells from the circulation two weeks after the initial labeling.
A preferential labeling of young cells by Cr$^{51}$ has been reported by some workers. Petrakis et al. (1958) and Walter et al. (1962) reported greater labeling of young cells in individuals with hemolytic disorders, but they were not able to demonstrate a similar difference in blood from normal individuals.

To determine the difference in Cr$^{51}$ labeling of younger and older red cells of normal individuals, Danon et al. (1966) used a specific gravity technique for separating these cells. He noticed that also in normal individuals the radioactivity of young cell population was higher than older cell population.

Disappearance of labeled red cells may be due to several processes giving rise to different forms of survival curve. In the case when cells are destroyed only due to senescence, and the labeled population of cells consists of equal numbers of cells of all ages, then the resultant curve should be linear.

Sheets et al. (1951) suggested the following formula for analysis of erythrocyte survival curves:

$$N_t = N_0 (1 - \frac{t}{T}) e^{-kt}$$

Where $N_t =$ number of cells present, or radioactivity due to them on day $t$

$T =$ average life span

$k =$ fractional daily loss

$N_0 =$ initial number of cells, or radioactivity count on day zero.

According to Mollison (1959) the model of the above formula assumes that the chromium survival curve is a result of at least two processes,
namely loss of red cells by aging, and loss of Cr\textsuperscript{51} by elution. The first process is linear, the second exponential or random. The random loss may be also defined as loss of either cell or label unrelated to the age of the cell and would thus include loss of chromium label due to elution.

Hughes-Jones and Mollison (1956) used a method of empirical correction to eliminate the effect of elution and to convert the chromium curve into the form of survival curve obtained by the differential agglutination method.

4. **Morphological Studies of Erythrocytes during Malarial Infection**

Physiological alterations leading to morphological changes and shortened erythrocyte survival have been observed in various hemolytic diseases such as sickle cell anemia, hereditary spherocytosis, elliptocytosis, and acanthocytosis (Neerhout, 1968). The occurrence of various physiological alterations in red blood cells during malarial infection leads one to speculate that morphological alterations of the erythrocytes occur as a result of direct or indirect action by the *Plasmodium* during penetration and intraerythrocytic growth. Most electron microscope studies of the malaria infected blood cells have been by thin section electronmicroscopy despite the fact that this technique does not yield a complete surface picture of an intact red blood cell.

Bahr (1969) studied certain physical properties of erythrocytes during malarial infection and confirmed some of the earlier reports of increases in the osmotic fragility, and volume of individual cells as infection progresses. He also examined normal and infected red blood
cell ghosts by use of the electronmicroscope and reported that infected cell membranes were smoother than those of uninfected cells which had a mottled appearance.

Ladda et al. (1969) studied the process of erythrocyte penetration by the merozoites of mammalian and avian malarial parasites. They indicated that during the process of penetration, the anterior conoid region of the merozoite has an important function, because only after a contact between the conoid region of merozoite and the blood cell membrane, is the process of penetration initiated. They also noticed that during the process of penetration the integrity of the blood cell membrane is not disrupted, rather the parasite moves from outside to inside of the cell by causing internal folding in the cell membrane. The folding is apparently caused by the interaction of a conoid secretion with the host cell membrane. After completion of the process of infolding fusion of the cell membrane at the site of penetration occurs. This helps to maintain the structural continuity of the cell membrane.

Trager et al. (1966) examined *P. falciparum* and *P. ovale* infected human red blood cells by a transmission electron microscope technique. They noticed a marked distortion of infected erythrocytes, and in erythrocytes of a *P. falciparum* infected human they noted that the uninfected erythrocytes showed multiple blister like abnormalities on certain portions of the cell membrane.

Lewis et al. (1969) used ion-etching and scanning electron microscopy to examine *P. knowlesi* infected monkey blood cells. In their unetched samples they noticed progressive distortion of the infected red
cells with growth of the parasites. The cells containing mature schizonts were particularly abnormal. The etched blood cells showed evidence of changes in internal structure corresponding to stages of parasite development.

Arnold et al. (1969) used transmission and scanning electron microscope techniques for morphological studies of malaria infected human and mouse blood cells. They noticed evidence of generalized swelling in most parasitized cells. This swelling caused deformity of the biconcave cells and led to formation of complete spheres. In addition they noticed the presence of cavities or holes in the surface of both parasitized and non-parasitized erythrocytes, which they indicated probably were caused by gelation of part of the red cell by some chemical action of the parasite during the process of penetration, and growth. The holes in the surface of some of the uninfected blood cells, in their opinion, were due to dislodgement of parasites from the infected cells.

Sprinz (1969) evaluated Arnold's work. He examined *P. cynomolgi* infected monkey blood cells using the scanning electronmicroscope. His observations differed from Arnold's as he could not find any holes on the surface of uninfected or infected blood cells. Trager (1969) also questioned the validity of Arnold's observations, specially the concept of parasite movement from one red blood cell to another during growth.

5. Survival Studies of Erythrocytes during Malarial Infection

After the introduction of isotopic tracing techniques erythrocyte survival studies were widely used to study the mechanisms and extent of
erythrocyte destruction during malarial infection.

Devakul and Maegraith (1959) transfused iron-59 tagged normal erythrocytes into monkeys infected with *P. knowlesi* and reported a fast removal of the tagged cells. However the rapid destruction they observed may have been due to infection of the cells.

Stohlman et al. (1963) studied the survival of autologous erythrocytes in humans during *P. cynomolgi* infection. They noticed shortening of the cell survival which was much more striking than was expected from the degree of parasitemia. In one case of *P. vivax* infection the shortening of the erythrocyte life span occurred even before the appearance of latent parasitemia. Zuckerman (1964) suggested that excessive destruction of apparently normal erythrocytes was due perhaps, to the presence of circulating anti-erythrocytic antibody.

George et al. (1966) examined the above hypothesis by studying erythrocyte survival of *P. berghei* infected blood after transfusing into normal rats. The transfused cells showed a normal survival pattern until the recipient rats became infected. Transfusion of sera from acutely malaria infected and immune rats into normal rats did not cause any decrease in the life span of normal erythrocytes. On the basis of these observations they concluded that in *P. berghei* infected rats erythrocyte destruction other than that caused by parasite emergence was caused by hypersplenism without any autoimmune component.

Kreier and Leste (1967) studied the effect of various levels of parasitemia on the survival of erythrocytes in rats. They transfused Cr51 labeled blood with various levels of parasitemia into compatible
Inbred rats and protected them from infection by chloroquine injections. The loss of $\text{Cr}^{51}$ in the first 24 hours correlated with the degree of parasitemia of the transfused blood, but as there was not any excessive erythrocyte destruction during subsequent period, they concluded that the non-parasitized blood cells of the infected animals were not damaged sufficiently to cause decreases in their life spans.

Kreier and Leste (1968) also studied the effect of infected host globulin on the survival of parasitized and non-parasitized erythrocytes. They reported that the globulin from the infected animal neither increased the efficiency of elimination of the parasitized erythrocytes nor caused a decrease in the survival times of non-parasitized erythrocytes.

Schachter (1968) studied the survival in normal birds of $\text{Cr}^{51}$ labeled chicken erythrocytes with various levels of parasitemia. He did not observe any relationship between degree of parasitemia and rate of erythrocyte destruction. This indicated cell damage and shorter life span of even non-parasitized erythrocytes of malarious chickens.

Writer and Kreier (1968) used a dual chromium$^{51}$ and di-isoproxyfluorophosphate ($\text{DFP}^{32}$) labeling technique to study the erythrocyte survival of normal and Plasmodium gallinaceum infected chickens. They noticed an accelerated erythrocyte destruction during the period preceding the detection of patent parasitemia. As the infection progressed they noticed faster disappearance of $\text{Cr}^{51}$ than of $\text{DFP}^{32}$ from the circulation. The disappearance of $\text{Cr}^{51}$ was greater than that expected if only parasitized erythrocytes were being destroyed, which suggested that during infection chromium$^{51}$ was lost at a faster than normal rate.
from both the parasitized and non-parasitized cells. The fast disappearance of chromium\textsuperscript{51} might have been due to an accelerated elution rate. It is known that chromium attaches to hemoglobin while DFP attaches to the erythrocyte membranes. As during malarial infection, various types of blood cell membrane abnormalities occur, causing increased fragility of both parasitized and non-parasitized cells; it is possible that loss of Cr\textsuperscript{51} might have been due to loss of hemoglobin from the cells. DFP\textsuperscript{32} being a non-elutable membrane label, only disappears after destruction of the cell. The DFP\textsuperscript{32} disappearance in their study did not indicate erythrocyte destruction, greatly in excess of that expected due to destruction of parasitized erythrocytes alone.

Kreier (1969) studied the effect of \textit{P. gallinaceum} infected chicken's plasma on the survival of normal erythrocytes. During his study neither incubation of erythrocytes from non-infected chickens in plasma from acutely infected chickens, nor injection of acutely infected chicken's plasma into normal chickens, caused decrease in the survival time of normal erythrocytes. The author suggested that like the plasma of \textit{P. berghei} infected rats, the plasma from chickens with acute \textit{P. gallinaceum} did not appear to contain auto-antibodies or soluble parasite or host products in quantities sufficient or of types able to cause destruction of erythrocytes of non-infected chickens.

6. \textbf{Physiological and Survival Studies of "Stress" Reticulocytes}

Under normal conditions blood of most adult animals contains between 0.5 to 4% reticulocytes which mature to be replacements for aged sequestered erythrocytes. These reticulocytes or young erythrocytes
contain a small amount of basophilic material and mitochondria and therefore take a bluish color when stained with most conventional polychrome stains, or with crystal violet. Within 33 hours of entry into the circulation the basophilic material disappears from the cells, indicating maturation of the erythrocyte. After maturation the erythrocytes function normally and survive the normal life span of erythrocytes.

During hemorrhagic "stress" caused by severe blood loss or erythrocyte destruction, the number of reticulocytes greatly increases in the circulation. The physiology and survival of the "stress" erythrocytes have been widely studied in different species of animals. Neuberger and Niven (1951) were the first to report that in the rabbit $^{15}$N tagged erythrocytes produced in response to acute blood loss survived for 38-48 days as opposed to the 61-65 day life span of erythrocytes of normal animals.

Berlin and Lotz (1951) studied survival of rat erythrocytes produced as a result of acute hemorrhage. They labeled the newly formed erythrocytes by injection of 20 μc of carbon 14 labeled glycine intraperitoneally 6 hours after removal of 5 ml blood from heart, and noticed a considerable decrease in the life span (mean life span - 27 days) of newly formed erythrocytes.

Stohlman (1961) studied the survival of "stress" reticulocytes produced in rats as a result of cardiac bleeding, phenylhydrazine, and erythropoietin treatment. He noticed significantly shortened life spans for red cells formed in response to severe anemia caused by blood loss or phenylhydrazine treatment. A similar decrease in life span was
also noticed in reticulocytes produced as a result of erythropoietin treatment. He also noticed greater shortening in the life span of erythrocytes produced at time of peak reticulocytosis than of cells produced during the stage when reticulocytosis was decreasing.

It is generally accepted that reticulocytes are slightly larger than mature red cells. But during hemorrhagic stress, abnormally large reticulocytes are found in the circulation. Brecher and Stohlman (1961) studied the size distribution of rat reticulocytes produced in response to anemia caused by phenylhydrazine treatment. They indicated that the reticulocytes produced during the first burst of erythroid regeneration were twice normal size. The degree of macrocytosis was related to the severity of the anemia and the initial crop of oversized cells were replaced by a successive crop of reticulocytes of more nearly size.

Similar observations were reported by Card and Valberg (1967) who studied "stress" reticulocytes in rabbits. The macrocytic cells produced in response to the administration of phenylhydrazine were of an average volume twice that of normocytic erythrocytes. They noticed that on the average 30% of the normocytes were destroyed in a random manner with a half time of 66 days, whereas macrocytes disappeared at two exponential rates, 25% with a half time of 4 days and 50% with a half time of 23 days. Their results suggest that at least two populations of short lived macrocytes are produced after stress and that they have a physical defect or metabolic anomaly that renders them more susceptible to destruction than normal cells.
Stryckmans et al. (1968) used an autoradiographic technique to study the life span of "stress" reticulocytes produced as a result of erythropoietic stimulation. They noticed that the reticulocytes produced as a result of mild erythropoietic stimulation mature into red cells with normal life spans; but the reticulocytes produced as a result of marked erythropoietic stimulation mature slowly and have abnormally short mean life-spans.

Robinson (1967) studied the rate of bilirubin production after transfusion of glycine-$^{14}$C labeled "stress" and normal reticulocytes into normal animals. He noticed a five fold greater $^{14}$C labeled bilirubin production in animals transfused with "stress" reticulocytes than in ones which received normal reticulocytes.

Observations similar to those of Robinson were also reported by Nagai and Kakishita (1969) and Robinson and Tsong (1970) regarding production of short lived erythrocytes during enhanced erythropoiesis. They noticed 4 to 6 fold increased destruction of "stress" reticulocytes in comparison with the normal reticulocytes.

Card et al. (1968) studied the process of autohemolysis and osmotic fragility of "stress" or macrocytic erythrocytes produced in response to hemolysis caused by phenylhydrazine treatment. They reported that the osmotic fragilities of normocytes and macrocytes were similar but that following 24 hours incubation at $37^\circ C$ in pH controlled serum autohemolysis of macrocytes was slightly greater than that of normocytes. They also noticed a marked increase in the autohemolysis and osmotic fragility of macrocytes under conditions of glucose deprivation and/or low pH.
Card et al. (1969) also studied the life-span and autohemolysis of macrocytic erythrocytes produced in rabbits in response to hemorrhage. Their erythrocyte survival study indicated that the macrocytic cells or "stress" reticulocytes disappeared at two rates with mean survival times of 3.1 and 28 days. These values are much less than the mean half life of normocytic cells. They also reported that under conditions of glucose deprivation autohemolysis of macrocytes was significantly greater than that of normocytes, and they considered that the shortened life-span and increased hemolysis of macrocytes in glucose free media are inherent characteristics of the erythrocytes produced in response to erythropoietic stress.

Sorbie and Valberg (1970) studied the splenic sequestration of stress erythrocytes in the rabbit. They reported that reticulocytes are sequestered by the spleen, liver and bone marrow but most of the sequestration of reticulocytes occurred in the spleen. They noticed greater and much longer sequestration of macrocytic reticulocytes than of normal reticulocytes. They suggested that selectively greater and longer sequestration of stress reticulocytes, in the unfavorable environment of the spleen might accelerate the destruction of these cells.

During malarial infection a large number of erythrocytes are destroyed, resulting in anemia and a marked increase in the number of immature erythrocytes or reticulocytes. As the erythrocytes produced during other conditions of anemic "stress" show a shortened life span, it is reasonable to expect that the erythrocytes produced under the severe anemic "stress" during malarial infection might also be abnormal.
and more prone to destruction. One of the purposes of this study was to determine the survival of erythrocytes produced during or following anemic "stress" caused by malarial infection.
MATERIALS AND METHODS

1. General Techniques

A. Experimental Animals and Parasites

The morphological studies of erythrocytes were conducted using adult albino Swiss mice, and Wistar rats six to ten weeks of age. For the erythrocyte survival studies inbred rats of Charles River Dunning Fisher Strain 344 were used. Animals were maintained in the Laboratory Animal Facility of the Microbiology Department. The principles of laboratory animal care of National Society for Medical Research were followed for the care and management of the animals.

The malarial parasite used during the study was *Plasmodium berghei*. The parasites were maintained in adult mice by continuous blood passage. For the various experiments the animals were infected by intraperitoneal injection of parasitized mouse blood cells.

B. Hematological Techniques

The course of the infection in the experimental animals was followed by daily determination of percent parasitemia, basophilia and packed cell volume (PCV) of blood samples collected after clipping of the tail. For the determination of parasitemia and basophilia, thin blood smears were prepared, dried in the air and fixed in absolute methyl alcohol for two minutes. The fixed blood smears were stained

---

for 45 minutes with 1:45 dilution of freshly diluted Giemsa stain in phosphate buffer, pH 7.2 (Appendix I). The stained blood smears were examined under an oil immersion objective and 100 to 200 blood cells, selected randomly, were examined for determination of percent parasitemia and basophilia.

The hematocrit, or percent packed cell volume (PCV), was determined by filling heparinized capillary tubes with blood, sealing them with seal-ease, and centrifuging them at full speed (approximately 12,000xG) in an International Clinical Centrifuge for five minutes. The hematocrit values were read with the help of an International Microcapillary reader.

2. Erythrocyte Morphological Studies

The blood samples for the morphological studies were collected from the normal and the infected animals during various stages of the infection. In mice generally erythrocytes were collected when the animals had low (15 to 25%), moderate (45 to 55%) and high (70 to 90%) parasitemia levels; whereas the blood samples from infected rats were collected during early (15 to 25% parasitemia), late (45 to 55% parasitemia), and recovery (parasitemia decreasing, reticulocytes increasing) stages of the infection.

\[2\] Chase Instrument Corp., Lindenhust, N. Y. - 11757

\[3\] Clay-Adams, Inc., New York, N.Y. - 10

\[4\] International Equipment Company, Boston, Mass.
A. Phase and Nomarski Interference Microscope Studies

Blood samples for the phase and interference microscope studies were collected from the heart, using dipotassium ethylenediamine tetraacetate (EDTA-Sequester-sol) as anticoagulant. The blood cells were washed thrice in phosphate buffered saline, pH 7.2 (Appendix I) and finally resuspended in phosphate buffered saline (PBS) containing one percent bovine serum albumin. Small drops of cell suspension were transferred to agar or gelatin coated glass slides and covered with glass cover slips. The cover slips were sealed on all sides with petroleum jelly to prevent evaporation of water from the wet mount preparation. The cells were examined with a Zeiss phase contrast and interference photomicroscope to observe the morphological differences between the blood cells of normal and parasitized animals, and the differences between the infected and noninfected cells in parasitized blood samples.

B. Light and Electron Microscope Studies of Fixed Blood Cells

Blood Collection, Fixation and Washing

To reduce the possibility of morphological changes during the process of washing, the blood was collected into a syringe containing 1% gluteraldehyde in PBS as fixative, and a few drops of EDTA as anticoagulant. The blood was allowed to remain in the syringe for approximately one hour for fixation to occur after which the erythrocytes were washed twice in PBS. After the initial washings the erythrocytes were resuspended in 1% gluteraldehyde solution and fixation was continued for

---

5 Cambridge Chemical Products Inc., 9182 Greenfield, Detroit, Michigan.
several more hours. After final fixation the erythrocytes were washed twice in PBS and thrice in distilled water.

**Light Microscope studies by the Giemsa-staining and Carbon-shadowing Techniques**

A one percent suspension of the fixed and washed blood cells was prepared in distilled water and a drop was transferred to a glass slide. The drop was allowed to dry slowly, after which the cells were stained with a 1:45 dilution of Giemsa stain in PB for half an hour followed by washing with distilled water. The stained slides were placed in a vacuum chamber and a thin layer of carbon was deposited from about a 45° angle over the slides. Cells so shadowed gave a three-dimensional picture on light microscope examination.

**Combined Giemsa-staining light microscopy and Electron Microscope Carbon-replica Techniques**

To obtain more accurate information about individual parasitized or nonparasitized cells, a combined Giemsa staining light microscopy and electron microscope carbon replica technique was developed. A one tenth percent suspension of the fixed and washed blood cells was prepared in distilled water and one drop of the suspension was transferred to a formvar coated 200 mesh (copper) electron microscope specimen grid. After drying, the blood cells were first stained with Giemsa, and then the grid was transferred to a slide, covered with a few drops of distilled water and a cover slide as a wet mount preparation. The cells were examined under the oil immersion objective of a photomicroscope and about ten to twenty squares of the grid were photographed. The location
of each photographed area was recorded on graph paper for easy and correct identification of the various areas during electron microscope examination of the carbon replica.

To obtain a replica of the erythrocytes surfaces, the grids were transferred to a vacuum chamber and first shadowed with platinum from a 25° angle after which carbon was deposited from a 90° angle. During the initial trials, it was noticed that during the washing procedure most of the replica over the erythrocyte surfaces fell, leaving only holes in place of cells. This was thought to be due to the formation of an incomplete carbon film around the cells. To prevent this collapse, carbon was deposited two more times on the preparations at a 25° angle, but each time from a different direction.

To dissolve the formvar, the grids were dipped in chloroform for 30 seconds, then removed and air dried. To remove the organic material, a fresh solvent solution was prepared by dissolving potassium permanganate and potassium dichromate in concentrated sulphuric acid (Appendix I). Two drops of this solvent solution were transferred to cups on a glass plate. The grid was floated on the surface of the solvent with the carbon film upwards. In one to two minutes the grids sank beneath the surface of solvent. The grids were then washed in distilled water to remove excess solvent solution. To remove inorganic crystals which would form on the grid from dried solvent solution, the grids were dipped in concentrated HCl for one minute and passed through three distilled water dippings. The grids were air dried and examined with a Hitachi model HS8 electron microscope.
Combined Giemsa-staining Light Microscopy and Scanning Electron-Microscope Technique

One drop of a one-tenth percent suspension of the fixed and washed blood cells was transferred to a glass cover slip. The cells were dried, stained with Giemsa, and then a few marked areas of the cover slips were photographed with a photomicroscope. The cover slips were then coated with gold and examined with a scanning electron microscope.

C. Morphological Studies of Rat Reticulocytes produced by Phenylhydrazine Hydrochloride Treatment

To study the morphology of reticulocytes produced as a result of anemia caused by processes other than malarial infection, rats were treated with phenylhydrazine hydrochloride. A solution of 10 mg of phenylhydrazine hydrochloride (PHC) in one ml of normal saline was prepared and two intraperitoneal injections at 48 hour intervals were given, using 3 mg PHC per 100 grams body weight. During the course of the treatment blood samples were examined to measure the hematocrit and percent reticulocyte values. Two blood samples, one when the reticulocytes were beginning to increase and another when there were about 25% reticulocytes, were collected by cardiac puncture. The blood cells were fixed and washed by the techniques described earlier and examined by the combined Giemsa-staining light microscopy and electron microscope carbon-replica technique.

D. Morphological Studies of Rat Reticulocytes produced by repeated Cardiac Bleeding

About three milliliters of blood was removed daily for three days from an adult rat. On the fourth day, when the reticulocytosis reached
about 20 percent, blood cells for the morphological studies were collected by cardiac puncture, fixed, washed and examined by the combined Giemsa-staining light microscopy and electron microscope carbon-replica technique.

3. Erythrocyte Survival Studies

A. Experimental Design

The experiment was designed to study the survival of malaria infected rat erythrocytes from animals in the stages of high parasitemia, early recovery and late recovery. In order to test the compatibility of the inbred strain of rats and accuracy of the transfusion, sampling and counting methods, a preliminary erythrocyte survival study was conducted with normal red blood cells.

To study the survival of erythrocytes from animals in the stage of high parasitemia, blood with about 50% parasitemia and 4% basophilia was collected from a rat, labelled with Cr$^{51}$ and transfused into compatible normal rats. To suppress infection in the recipient rats the animals were treated daily with 5 mg of chloroquine phosphate$^6$ and 9 mg of sulfadiazine$^7$ per 100 gm body weight.

To study the survival of erythrocytes from animals in an early stage of recovery, young rats were infected with Plasmodium berghei and when parasitemia reached about fifty percent, the rats were treated with a daily dose of 2 mg chloroquine phosphate and 6 mg sulfadiazine per 100 grams body weight. During the recovery period a sharp increase in the

---

$^6$City Chemical Corp., New York.

number of nonparasitized reticulocytes was noticed. Blood from the recovering rats containing about 80 to 90 percent nonparasitized reticulocytes was labeled with Cr$^{51}$ and transfused into normal compatible inbred rats. The recipient rats were treated with a daily dose of 2 mg chloroquine phosphate and 6 mg of sulfadiazine per 100 gm body weight throughout the period of the study, and Giemsa stained blood smears were examined and PCV recorded to determine if the rats developed an infection.

To study the survival of erythrocytes from animals in a late stage of recovery, P. berghei infected rats were treated with antimalarial drugs when parasitemia reached about 50%. The animals were allowed to recover completely and then fifteen days after disappearance of parasitemia blood was collected from the rats, labeled with Cr$^{51}$ and transfused into normal compatible rats. At the time of blood collection the packed cell volume and basophilia in the donor rats were normal and parasitemia was absent.

B. Experimental Techniques

**Labeling of Erythrocytes with Chromium (Na$_2$Cr$^{51}$O$_4$)**

Three to four ml of blood was collected by cardiac puncture from each donor rat using EDTA as anticoagulant. The blood cells were washed thrice with PBS by centrifugation at 750 g force for five minutes in a Sorvall RC-2 refrigerated centrifuge. Thereafter the blood cells were incubated for half an hour at 37°C with Na$_2$Cr$^{51}$O$_4$ using 30 μc of radioactivity for each ml of packed red blood cells. During the incubation period the blood cells were occasionally stirred. After incubation the

---

8 Chromatope Sodium, E. F. Squibb & Sons, Inc., N. Y.
cells were washed thrice in PBS to remove unbound isotope, and finally the packed blood cells were resuspended in a volume of PBS equal to the volume of packed cells. About one ml of the labeled blood cell suspension was transfused into recipient rats by intracardial injection.

**Sampling of Blood**

The first sample of blood was collected within half an hour after the blood transfusion, thereafter the samples were collected daily for first few days. Later the blood samples were collected two or three times a week over one to four week periods.

Blood sampling was done by clipping the tail and filling each of two pre-weighed heparinized capillary tubes about two-thirds full. The capillary tubes were weighed again and the exact weight of each blood sample calculated. The blood from one of the capillary tubes was transferred to a tube containing two ml of distilled water. The other capillary tube was first centrifuged and the FCV measured, then its contents was added to the distilled water also. All samples were stored in a freezer until the end of the sampling period.

**Counting and Data Processing**

All samples from an individual group of rats were counted on the same day in a thallium-activated solid sodium-iodide crystal well-type scintillation counter. Five one-minute counts were made on each sample. After substracting background count, the counts per minute (CPM) were determined for each sample. The counts were adjusted to a standard weight of 0.1 gm of blood cells by taking into consideration the FVC and weight of individual blood samples. Finally the counts were expressed
in terms of percentage by considering the zero time (first sample) count as one hundred percent and expressing all other samples as a percent of zero time count.
RESULTS

1. General Course of *P. berghei* infection in Mice and Rats

In spite of overall similarity the course of *P. berghei* infection differs to a considerable extent in mice and rats. Therefore it is desirable to discuss the course of plasmodial infection in individuals of each of these species before going into the details of the effects of plasmodial infection upon erythrocyte morphology.

The course of *P. berghei* infection in mice may vary depending on various factors such as the number of parasites introduced into the animal, animal age, breed and other factors but the general pattern is similar in all mice (Figure 1). Figure 1 was drawn from average data of five infected mice. The mice were examined daily after intraperitoneal injection of about $2 \times 10^6$ parasitized erythrocytes. In mice the parasitized blood cells appear in the peripheral circulation usually on the second day after infection and thereafter their number increases regularly until in four to five days about 80 to 90 percent of the erythrocytes are parasitized. During the same period there is destruction of a large number of cells which causes the packed cell volume to decrease considerably. The decrease in packed cell volume stimulates production of basophilic erythrocytes, but the animal generally dies before basophilia reaches a high level.

The course of *P. berghei* infection in rats depends to a great extent on the animals age. In young rats the infection follows a
Figure 1. Showing the usual course of *P. berghei* infection in adult mice after intraperitoneal injection of $2 \times 10^6$ parasitized erythrocytes. The figure was constructed from average data (Table 1) of five mice.
Parasitemia
Packed Cell Volume
Basophilic Erythrocytes

Days After Infection

Figure 1
course similar to that in mice, but in older rats the disease takes a different course (Figure 2). The duration of the prepatent period depends on the infective dose, larger doses cause a shorter prepatent period. The parasites appear in the circulation two or three days after infection and then their number gradually increases until in the next five or six days about 50 to 60% of the blood cell population is infected. During this period the packed cell volume decreases to less than half the normal value. The decrease in the packed cell volume initiates events which cause the stimulation of the production of basophilic erythrocytes. As the parasites have affinity for basophilic or immature red blood cells, most of the basophilic cells produced during this period also get infected. After peak parasitemia is reached recovery normally occurs. The first indication of recovery is a decrease in the number of parasitized blood cells. During the early part of the recovery period a sharp increase in the number of unparasitized basophilic erythrocytes also occurs. Basophils may make up 80 to 90% of the blood cell population. Production of basophilic erythrocytes causes an increase in the packed cell volume. During the later part of the recovery period the packed cell volume returns to normal and the number of basophilic erythrocytes present decreases toward normal.

2. Light microscope observations of Mouse and Rat erythrocytes during *P. berghei* infection

A. The appearance of Giemsa stained erythrocytes

Photomicrographs of Giemsa stained erythrocytes from mice and rats in various stages of *P. berghei* infection are presented in Figures 3 and 4.
Figure 2. Showing the usual course of *P. berghei* infection in adult rats after intraperitoneal injection of $2 \times 10^6$ parasitized erythrocytes.

The figure was constructed from average data (Table 2) of five rats.
Parsitemia
Packed Cell Volume
Basophilic Erythrocytes
Infected Basophilic Erythrocytes

FIGURE 2
respectively. Figure 3A is a photomicrograph of normal uninfected mouse erythrocytes. Figures 3B, C and D are photomicrographs of blood cells from animals with low (15 to 25%), moderate (45 to 55%), and high (70 to 90%) levels of parasitemia respectively. During a severe infection parasites become so numerous in the blood that many cells get multiple infections (Figure 3D) and many free parasites occur in the blood (arrow "p" Figure 3C and D). In the infected cells as the parasite matures it uses hemoglobin and the cell morphology changes (Figure 3D). The erythrocytes containing small parasites appear normal ("n") whereas the cells with larger parasites ("m") appear hypochromic and large in size.

Figure 4A is a photomicrograph of Giemsa stained normal rat erythrocytes. Like normal mouse blood cells, normal rat blood cells are discoid and are pale in the center because of their biconcave shape. There is little variation in the size of normal blood cells and even basophilic cells (arrow "b") appear to be approximately the size of mature blood cells. Figures 4B and 4C are photomicrographs of blood cells from rats in early (15 to 25% parasitemia) and late (45 to 55% parasitemia) stages of infection. In spite of severe infection no marked increase in the size of the erythrocytes (Figure 4C) occurs.

Figure 4D is a photomicrograph of Giemsa stained erythrocytes from a rat in an early stage of recovery. A number of basophilic erythrocytes or reticulocytes (arrows "b") are visible. As P. berghei has a preference for immature blood cells, a higher proportion of reticulocytes than mature erythrocytes are infected. The large size of these infected
Figure 3. Photomicrographs of Giemsa stained erythrocytes from mice in various stages of malarial infection. (A) Normal. (B) Low (15 to 25%) parasitemia. (C) Moderate (45 to 55%) parasitemia. (D) High (70 to 90%) parasitemia. Even when there is high parasitemia the erythrocytes ("n") containing small parasites appear normal whereas those containing large parasites ("m") appear pale (hypochromic) and large in size. Arrows "p" indicate free parasites.
Figure 4. Photomicrographs of Giemsa stained erythrocytes from rats in various stages of malarial infection and recovery. (A) Normal mature erythrocytes and a normal basophilic erythrocyte (cell "b"). (B) Early parasitemia (15 to 25%). (C) High parasitemia (45 to 55%) and no basophilia. (D) High parasitemia (60%) and high (50%) basophilia. Notice the large size of the infected basophilic erythrocytes (arrows "b") in comparison to the infected mature erythrocytes (arrows "m"). (E) Recovery stage, parasitemia absent but 90% basophilic erythrocytes of macrocytic form. (F) Late recovery stage, 70% basophilic erythrocytes.
FIGURE 4
reticulocytes is apparent in the photomicrograph, they are not only bigger than the infected mature cells (arrow "m") but are also bigger than non-infected basophilic cells (cell "b" Figure 4A).

Figure 4E is a photomicrograph of Giemsa stained erythrocytes from a rat in the late stages of recovery after disappearance of parasites from the blood. Most of the cells are reticulocytes which were produced during severe anemic stress and which are macrocytic in nature. As recovery continues the number and size of reticulocytes in the circulation slowly decreases (Figure 4F).

B. The appearance of fresh blood cells by Phase Contrast and Nomarski Interference Microscopy

Figures 5 and 6 are photomicrographs of mouse erythrocytes taken by phase contrast and interference microscopy. Erythrocytes of normal mice (Figure 5A and 6A) and erythrocytes from mice with low (Figure 5B and 6B), moderate (Figure 5C and 6C) and high (Figure 5D and 6D) levels of parasitemia are shown. Most of the normal mouse blood cells (Figures 5A and 6A) are biconcave disks. Changes in the shape of the parasitized blood cells occur already during the early and moderate stages of parasitemia. The most common change in the parasitized cells is the assumption of a signet ring form (arrows, Figure 5B and 6B). The type and degree of change in shape is determined by the size and location of the parasite in the cell. In photomicrograph 5C the cell containing a small parasite ("a") appears almost normal whereas cells containing larger parasites ("b" and "c") are more severely altered.
Figure 5. Photomicrographs by the phase contrast technique of erythrocytes taken from mice during various stages of malarial infection.
(A) Normal. (B) Low parasitemia (15%), arrows indicate infected erythrocytes in signet ring form. (C) Moderate parasitemia (50%), cells "a", "b" and "c" show that with increasing parasite size there is increased morphological alteration. (D) High parasitemia (90%), both infected ("p") and non-infected ("n") erythrocytes appear to be converted to spherocytes.
Figure 6. Nomarski interference photomicrographs of erythrocytes from mice in various stages of malarial infection. (A) Normal. (B) Low parasitemia, arrow indicates parasitized erythrocyte in signet ring form. (C) Moderate parasitemia. (D) High parasitemia, both infected ("p") and non-infected erythrocytes ("n") appear to be converted to spherocytes.
During periods when there is a high level of parasitemia (Figures 5D and 6D) changes in the blood cell shape become more distinct and most of the parasitized cells ("p") as well as the nonparasitized cells ("n") become spherical in shape.

In photomicrographs made by the interference technique the blood cells appear three dimensional and the parasites appear to stand above the cell surface (arrow, Figure 6D). This is probably an illusion caused by the optics of interference microscopy because no such thing was observed when the fixed cells were examined following Giemsa-staining and carbon shadowing.

Figures 7 and 8 respectively are phase contrast and interference microscope photographs of rat erythrocytes. Normal rat blood cells are shown in photomicrographs 7A and 8A which, like normal mouse cells, are round biconcave disks. Photomicrographs 7B and 8B show the blood cells from rats in an early stage of infection. The infected cells (arrow) appear swollen or signet ring shaped but the uninfected cells still have their normal biconcave shape. As the degree of parasitemia and the size of the individual parasites in the infected cells increases, the changes in the morphology of the infected erythrocytes become more prominent (Figure 7C and 8C). During this stage of infection in which there is rising parasitemia most of the parasitized erythrocytes (arrows) appear spherical, but the uninfected cells still maintain their normal shape.

Photomicrographs of erythrocytes from rats in the recovery stage of the infection are shown in Figure 7D and 8D. Most of the cells present during the recovery period are reticulocytes or immature
Figure 7. Photomicrographs by phase contrast of erythrocytes from rats in various stages of malarial infection. (A) Normal. (B) Low (15%) parasitemia, arrow indicates an infected erythrocyte with signet ring shape. (C) High (50%) parasitemia, arrows indicate spherocytic infected erythrocytes. (D) Late Recovery (parasitemia absent, basophilia 70%), large and ring shaped basophilic erythrocytes (arrows "r") and normal, biconcave mature erythrocytes (arrows "m") are present.
Figure 8. Photomicrographs by the Nomarski interference technique of rat erythrocytes showing various effects of parasitization. (A) Normal. (B) Low parasitemia (15%), arrow indicates an infected erythrocyte having signet ring shape. (C) High parasitemia (50%), arrows indicate infected erythrocytes having spherocytic shape. (D) Early recovery (parasitemia 40%, basophilia 50%), large spherocytic parasitized basophilic erythrocytes are visible.
basophilic erythrocytes. In the photomicrograph made by interference microscopy (8D), erythrocytes from a rat in the early stage of recovery are shown. These reticulocytes are large in size, and the parasitized ones have assumed a spherocytic form. The photomicrograph made by the phase contrast technique (7D) shows erythrocytes from rats in a later stage of recovery, after disappearance of parasites from the circulation. The reticulocytes (arrows "r") are bigger than the mature erythrocytes (arrow "m") and instead of being biconcave disks, they appear to be flattened and ring shaped.

C. The appearance of Giemsa stained and Carbon Shadowed gluteraldehyde fixed erythrocytes

The appearance of gluteraldehyde fixed mouse and rat erythrocytes which have been Giemsa stained and carbon shadowed may be seen in the photomicrographs shown in Figures 9 and 10. Due to the carbon shadowing the biconcave disk shape of the erythrocytes is quite distinctly visible. The immediate fixation of the erythrocytes which resulted from collection of the blood directly into a gluteraldehyde containing solution has helped to maintain the original shape of the erythrocytes even after drying and shadowing in the vacuum chamber.

Figures 9B, C, and D are photomicrographs of mouse blood cells with low, moderate and high levels of parasitemia respectively. In photomicrograph 9B the infected erythrocytes appear oval and signet ring shaped (arrows). As the parasitemia increases, the proportion of distorted cells increases until finally when there is a high level of parasitemia (Figure 9D) and almost all the cells appear altered.
Figure 9. Photomicrographs of carbon shadowed Giemsa stained mouse erythrocytes. (A) Normal. (B) Low (20%) parasitemia. (C) Moderate (40%) parasitemia. (D) High (70%) parasitemia. Parasitized erythrocytes are swollen and distorted (arrows), the greater swelling appears to be where the parasite is located.
Figure 10. Photomicrographs of carbon shadowed Giemsa stained rat erythrocytes. (A) Normal. (B) Low (20%) parasitemia. (C) High (40%) parasitemia, arrows indicate spherocytic infected erythrocytes. (D) Early recovery, arrows indicate large basophilic erythrocytes.
Photomicrographs 10B and 10C are of rat erythrocytes with low and high levels of parasitemia respectively. By the carbon shadowing technique as by the phase and interference microscope techniques only the infected erythrocytes (arrows) appear swollen or spherical whereas the uninfected cells appear normal in shape. Blood cells including reticulocytes (arrows) from a rat in the recovery stage of the disease are shown in Figure 10D.

3. Appearance of Mouse erythrocytes during *P. berghei* infection

A. The combined technique using Giemsa staining and Light Microscopy followed by Electron Microscopy of carbon replicas of the previously examined erythrocytes

A standard carbon replication technique does not permit one to determine which erythrocytes are parasitized. Therefore to gather information in regard to the morphology of individual infected or non-infected erythrocytes, the cells were examined using a combined Giemsa staining light microscopy and electron microscope carbon replica technique which permitted identification of individual parasitized erythrocytes.

Figures 11, 12, 13, and 14 are micrographs of normal and parasitized mouse blood cells with low, moderate and high levels of parasitemia respectively. Photomicrographs of the cells after Giemsa staining are shown in Figures 11A, 12A, 13A and 14A and electromicrographs of the same cells taken after carbon replication are shown in Figures 11B, 12B, 13B, and 14B. Additional electromicrographs of carbon replicas of mouse erythrocytes with inserts of the photomicrographs of the same cells after Giemsa staining are shown in Figure 15.
Figure 11. Photomicrographs of Giemsa stained normal mouse erythrocytes taken by light microscopy (Figure A) and electron micrographs of carbon replicas of the same erythrocytes (Figure B). Non-infected normal mature mouse erythrocytes are fairly uniform biconcave disks with smooth surfaces. A basophilic erythrocyte (arrow, Figure 11A) has a pitted surface however (arrow, Figure 11B).
Figure 12. Photomicrographs of erythrocytes of a mouse infected with malaria (20% parasitemia). Light microscopy of a Giemsa stained preparation (Figure A). Electron microscopy of carbon replica of the same erythrocytes (Figure B). Non-infected erythrocytes appear normal whereas infected erythrocytes in the same blood (arrows, Figure A) appear swollen in the region where the parasite is located (arrows, Figure B).
Figure 13. Photomicrographs of Giemsa stained erythrocytes (Figure A) of a mouse with malaria (50% parasitemia) and electronmicrographs of the same erythrocytes after carbon replication (Figure B). The carbon replicas reveal that parasites have various effects on the cells morphology. Erythrocyte "a" in spite of parasitization appears normal, while in the same field, another parasitized cell "b" shows a small depression (arrow) in the region of parasite location. Parasitized cell "c" has assumed a signet ring shape and cell "d" which contains large parasites is grossly distorted, enlarged and swollen in the region of the parasite.
Figure 14. Photomicrographs of erythrocytes of a mouse with malaria (70% parasitemia). Light microscopy of a Giemsa stained preparation (Figure A) and electron microscopy of carbon replicas of the same erythrocytes (Figure B). Erythrocytes with various morphological abnormalities such as a twisted appearance (cell "a"), a granular surface (cell "b"), or a spherocytic form (cell "c") become common during the stage of high parasitemia. Cell "b" which is not parasitized is a basophil. Cells "a", "d" and "e" are parasitized.
Normal mouse erythrocytes are shown in Figures 11A and B and 15A.
Most of the mature blood cells are biconcave disks and have smooth surfaces. One erythrocyte (arrow) shown in Figure 11B has a pitted surface. This erythrocyte is revealed to be an immature basophilic erythrocyte (arrow) in the photomicrograph of the Giemsa stained preparation (11A).

During the early stages of infection most of the uninfected erythrocytes (Figure 12, 15B) in blood of infected animals appear normal and resemble in appearance erythrocytes in normal mouse blood (Figure 11, 15A). Even during the early stages of infection however parasitized erythrocytes show morphological alteration in the form of some swelling in the region where the parasite is located which commonly changes the cell shape to a signet ring form. (Arrows, Figures 12, 15B)

Erythrocytes with a moderate level of parasitemia (45 to 55%) are shown in Figures 13 and 15 C. At this stage of infection, parasites of various sizes may be present in the erythrocytes. Not all parasitized erythrocytes have the same degree of morphological alteration. For example, in Figure 13 infected cell "a" appears to be almost normal, whereas the cell "b" contains only a small depression (arrow) in the region where the parasite is located. On the other hand some parasitized cells are greatly distorted. Parasitized cell "c" has a signet ring form, and cell "d" which contains a large schizont appears to be swollen where the parasite is located. In Figure 15C also a parasitized cell ("a") is swollen in the region where the parasite is located but in the same figure (15C) the erythrocyte membranes of three other cells ("b", "c" and "d") appear to sink in where they cover the parasite.
Figure 15. Electron micrographs of carbon replicas of mouse erythrocytes and photomicrographs of Giemsa stained preparation of the same erythrocytes before replication (inserts). (A) Normal mouse erythrocytes. (B) Erythrocytes of a mouse with 20% parasitemia. A parasitized erythrocyte (arrow) has a membrane sunken in the region of the parasite. (C) Erythrocytes of a mouse with 50% parasitemia. An erythrocyte ("a") is swollen whereas other erythrocytes ("b", "c" and "d") are sunken in the region of parasite. (D) Erythrocytes of a mouse with 70% parasitemia. Erythrocytes with distorted (cell "b") or spherocytic (cell "c") form and rough membranes occur commonly during the stage of high parasitemia.
Erythrocytes from a mouse with parasitemia of 70 to 90% are shown in Figures 14 and 15D. At this stage of the infection many irregularly shaped erythrocytes are found in the circulation. For example infected cell "a" of Figure 14 appears to be so twisted that the central depression has become an irregularly shaped fold. In addition many erythrocytes have a granular membrane (cells "b", Figure 14B, 15D) and are spherocytic (cells "c", Figure 14B, 15D). Noninfected erythrocytes with granular surfaces such as "b" in Figure 14 are probably reticulocytes.

B. The combined technique using Giemsa staining and Light Microscopy followed by Scanning Electron Microscopy of the same erythrocyte

In Figure 16 photomicrographs A, C, and E are of Giemsa stained normal and infected mouse erythrocytes. Scanning electron micrographs B, D and F show the same erythrocytes after gold coating.

Figure 16B is a scanning electron micrograph of normal mouse erythrocytes. Except for cell "a" most of the cells are concave disks. Cell "a" is spherocytic and has a pitted surface. Examination of the photomicrograph (16A) reveals that cell "a" is a basophilic erythrocyte.

Figure 16D is a scanning electron micrograph of erythrocytes from a mouse with a moderate (40%) level of parasitemia. Till this stage of the infection most of the nonparasitized erythrocytes in the blood of the infected mice look normal while the infected blood cells have various morphological abnormalities. Even at this parasitemia some parasitized erythrocytes ("a") are almost normal or are only slightly modified. Cells "b" and "c" have small holes in the membrane where the
Figure 16. Photomicrographs of normal and infected mouse erythrocytes after Giemsa staining (Figure 16A, C, and E) and scanning electron micrographs of the same erythrocytes (Figure 16B, D, and F). Photomicrograph (A) and scanning electron micrograph of normal mouse erythrocytes (B). All the cells are fairly uniform, biconcave disks except the basophilic erythrocyte ("a") which is spherocytic and has a pitted surface. Erythrocytes from a mouse with 40% parasitemia (C) and (D). The non-infected cells appear normal but some of the infected cells ("b", "c" and "d") show small "holes" and other membrane abnormalities of various magnitude. Erythrocytes from a mouse with 90% parasitemia (E) and (F). Most of the cells are distorted and have a rough and irregular surface (arrows).
parasite lies. Erythrocyte "d" (Figure 16C and D) contains several parasites. Its membrane is abnormal and appears to have a hole.

Figure 16E and F are micrographs of erythrocytes of mice with high (90%) parasitemia. In the photomicrograph of the Giemsa stained erythrocytes (Figure 16E) most of the cells can be seen to be parasitized. In the scanning electron micrograph these cells are seen to be distorted and to have irregular surfaces. The cells also appear flat and what would correspond to the "holes" seen in the erythrocytes in Figure 16D are seen to be shallow cavities and depressions in the erythrocyte surface (arrows).

4. The appearance of erythrocytes from Rats with *P. berghei* infections

   A. The combined technique of Giemsa staining and Light Microscopy followed by Electron Microscopy of carbon replicas

   Electronmicrographs of normal and *P. berghei* infected rat erythrocytes are presented in Figures 17 and 18. Each figure has a photomicrograph of the same erythrocytes stained with Giemsa as an insert. Figures 17A and 18A are micrographs of mature erythrocytes from a normal non-infected rat. These erythrocytes can be seen to have a concave disk shape and a smooth surface free of pits and irregularities.

   Micrographs of carbon replicas of erythrocytes from rats in an early stage of malarial infection (15 to 25% parasitemia) are shown in Figures 17B and 18B. The parasitized blood cells are altered morphologically. The larger the parasite the greater is the alteration. For example cell "a" of Figure 17B contains a small parasite and is almost normal whereas cell "b" containing a bigger parasite is swollen in the
Figure 17. Electron micrographs of carbon replicas of rat erythrocytes and photomicrographs made before replication of the same erythrocytes stained with Giemsa (inserts). (A) Mature erythrocytes from a normal non-infected rat. (B) Early infection; cell (arrow "a") containing a small parasite appears normal whereas the infected cell (arrow "b") containing a large parasite appears slightly swollen. (C) Peak infection; cell (arrow "a") containing a schizont or large parasite is spherocytic and has a rough membrane, non-parasitized cells in the same field (arrows "b") are essentially normal. Another parasitized cell (arrow "c") is only slightly distorted. (D) Recovery period; large, flat and distorted immature basophilic erythrocytes are present in the blood.
Figure 18. Electron micrographs of carbon replicas of rat erythrocytes and photomicrographs made before replication of the same erythrocytes stained with Giemsa stain (inserts). (A) Mature erythrocytes from a normal non-infected rat. (B) Early infection. (C) Peak infection. The parasitized erythrocytes (arrows "a") are spherocytic and have rough membranes. Most of the non-infected erythrocytes (arrows "b") appear essentially normal. (D) Recovery period; large immature basophilic erythrocytes which are distorted and have pitted surfaces.
region where the parasite is located. An erythrocyte containing a large parasite may become spherocytic (cells "a" Figure 18B) although non-parasitized erythrocytes in the same blood sample appear normal (Figure 18B, arrow "b").

Micrographs of erythrocytes from rats at peak (50%) parasitemia are shown in Figures 17C and 18C. During this stage of infection the proportion of erythrocytes containing large parasites and schizonts is large and generally these cells are altered morphologically. For example cells "a" of Figures 17C and 18C contain large parasites or schizonts and they are irregular in shape or spherocytic and have rough surfaces. An infected cell (arrow "c") containing a small parasite (Figure 17C) is signet ring shaped. In the rat, even during the stage of high parasitemia, most of the morphologically altered erythrocytes are those which are parasitized. The non-infected erythrocytes (Figure 17C and 18C, arrows "b") from the same blood samples appear completely normal.

If during peak parasitemia rats are treated to prevent their dying of infection, or if they recover naturally, a large proportion of basophilic erythrocytes are found in the circulation. Carbon replicas of these basophilic cells are shown in Figures 17D and 18D. These cells are large and irregular in shape and they have pitted surfaces.

B. The combined techniques of Giemsa staining and Light Microscopy followed by Scanning Electron Microscopy

The appearance of Giemsa stained rat erythrocytes by light microscopy and the appearance of the same erythrocytes by scanning electron
microscopy is shown in Figure 19. Photomicrographs A, C, and E are of the Giemsa stained cells, whereas B, D, and F are by scanning electron microscopy. Figure 19A and B show normal rat erythrocytes, C and D erythrocytes from rats with a patent parasitemia and E and F erythrocytes from a recovering rat.

In scanning electron micrograph 19B the erythrocytes of the normal rat resemble the normal erythrocytes seen earlier by various light and electron microscope techniques.

In the infected blood sample (Figure 19C, D) most of the uninfected erythrocytes appear normal in shape, but the infected erythrocytes show various degrees of morphological alteration. For example in Figure 19D infected cell "a" containing a small parasite appears almost normal, whereas neighboring cell "b" containing multiple parasites has a bulge over one of the parasites. A third infected cell ("c") has a small surface depression in the region where one of the parasites is located.

Erythrocytes from a rat in the recovery stage of plasmodial infection are shown in Figures 19E and F. The blood sample contained about eighty percent immature basophilic erythrocytes. Most of these cells are large and irregular and they have pitted surfaces. This type of pitted surface does not occur in mature normal (Figure 19B) or infected (Figure 19D) erythrocytes.

5. Morphological observations of Rat reticulocytes produced following Phenylhydrazine treatment

Phenylhydrazine hydrochloride acts as an erythrocyte membrane poison and causes rapid destruction of red blood cells following its injection
Figure 19. Photomicrographs of erythrocytes from normal, infected and recovering rats taken by light microscopy after Giemsa staining (A, C, and E) and scanning electron micrographs of the same erythrocytes after gold coating (B, D, and F). (A and B) Non-infected mature rat erythrocytes. (C and D) Erythrocytes from a malaria infected animal. The non-infected and some of the infected erythrocytes (arrow "a") appear almost normal whereas most of the infected erythrocytes (arrow "b" and "c") have some morphological abnormality. (E and F) Immature basophilic erythrocytes from a rat recovering from malaria infection. Most of the cells are distorted and have a pitted surface.
into an animal. Destruction of erythrocytes leads to a rapid influx into the circulation of immature basophilic erythrocytes or reticulo­cytes. To determine whether the reticulocytes produced during the recovery stage of malarial infection were abnormal due to the disease or due to their being of reticulocyte nature it was decided to study the morphology of reticulocytes produced following phenylhydrazine treatment.

Figure 20A is an electron micrograph of a carbon replica of rat erythrocytes collected two days after intramuscular injection of phenylhydrazine into an adult rat. At that stage the number of basophilic blood cells in the circulation was just beginning to increase. The cells are distorted in shape, probably due to the toxic effect of phenylhydrazine. The erythrocytes shown in Figure 20B were collected two days after the first sample, when the number of basophilic erythrocytes in the circulation was about twenty-five percent. These newly produced basophilic erythrocytes (arrows) are swollen and have a pitted surface.

Reticulocytes produced following phenylhydrazine treatment are pitted and irregular in appearance as are other reticulocytes but as phenylhydrazine itself is an erythrocyte membrane poison it is unclear whether these abnormalities are due to the effect of the drug or are simply characteristics of basophilic blood cells. To answer this question it was decided to study reticulocytes produced following repeated cardiac bleeding.
Figure 20. Carbon replicas of erythrocytes of a rat injected with phenylhydrazine. Erythrocytes which have been damaged by the drug are shown in Figure 20A. In Figure 20B are shown replicas of two replacement basophilic erythrocytes (arrows).
6. Morphological characterization of Rat reticulocytes produced following repeated Cardiac bleeding

Figure 21 is a photomicrograph of a carbon replica of rat erythrocytes collected following repeated cardiac bleeding. The proportion of basophilic erythrocytes in the circulation was about twenty percent. In the figure two normal erythrocytes and a basophilic erythrocyte are shown. The basophilic erythrocyte (arrow) appears dark in the photomicrograph of the Giemsa stained preparation (insert). In the carbon replica this cell can be seen to have a pitted and irregular surface.

7. Survival of Rat Erythrocytes labeled with Chromium$^{51}$ (Cr$^{51}$) and transfused into Normal Animals

A. Normal Erythrocytes

The data obtained after transfusion of normal Cr$^{51}$ labeled rat blood into four normal homologous animals is given in Table 5. The same data is presented graphically in Figure 22. Chromium label elutes from the labeled blood at a fairly constant rate. The decline in radioactivity of the normal transfused blood is caused by loss of erythrocytes due to senescence and random processes including elution of label, loss by sample collection, and bleeding after sample collection.

To estimate the Cr$^{51}$ loss due to random processes the following formula was used

$$N_t = N_0 \left(1 - \frac{t}{T} \right) e^{-kt}$$

Where $N_t$ = radioactivity of labeled cells on day $t$

$t$ = time measured in days

$T$ = average life span of erythrocytes in days
Figure 21. Carbon replicas of erythrocytes of a rat bled repeatedly from the heart. The membrane of the basophilic cell (arrows) is pitted and rougher than that of the mature erythrocytes.
Figure 22. Survival of erythrocytes transfused from a normal inbred rat to compatible inbred non-infected rats. Curve (actual curve) was drawn from average data from four rats. This curve closely matches a theoretical curve (derived curve) calculated using 54 days as the erythrocyte life span and a daily random erythrocyte loss of 6.5% of the remaining label.
FIGURE 22

% Cr remaining

-actual curve

- derived curve

DAYS

0 5 10 15 20

0 20 40 60 80 100
\( k = \) rate constant describing daily loss of label (Cr\(^{51}\)) due to elution, and other random processes

\( N_0 = \) Radioactivity on day 0.

By using 54 days as the average rat erythrocyte life span (T) (Owen and Orvis, 1966) and various k values, it was determined that a curve generated using "k" value of 6.5% of the remaining label per day fitted our data on normal erythrocyte survival fairly well (Figure 22).

B. Infected Erythrocyte (50% parasitemia)

Table 6 shows the percent of Cr\(^{51}\) remaining over a period of about one week in recipient rats, transfused with Cr\(^{51}\) labeled blood with 50% parasitemia. The infected erythrocyte survival curve is presented in Figure 23 along with a normal erythrocyte survival curve. There is a rapid loss of Cr\(^{51}\) label in the first 36 hour period after transfusion of the parasitized blood, which probably is due to the destruction of the parasitized blood cells. In the next 36 hours the rate of Cr\(^{51}\) loss slows down, and thereafter the curve follows a pattern which is nearly similar to the survival curve of normal erythrocytes. The relationship between the two curves is clearly visible when one calculates the ratio of the infected to normal blood curves. In the first 3 days after transfusion the ratio between the infected and normal survival curves dramatically changes but thereafter the ratio remains almost constant at a value of about 0.5. This indicates that after initial destruction of parasitized erythrocytes, the remaining erythrocytes are behaving as normal blood cells do and are destroyed at a rate similar to that of normal erythrocytes.
Figure 23. Survival of erythrocytes transfused from *P. berghei* infected rats (50% parasitemia) to compatible drug treated non-infected rats. The infected erythrocytes survival curve was drawn with average data from three transfused rats. After an initial drop, attributable to the destruction of parasitized erythrocytes, the curve runs almost parallel to the normal erythrocyte survival curve. The ratio of Cr\(^{51}\) remaining at various intervals after transfusion of parasitized blood to Cr\(^{51}\) remaining after transfusion of non-infected blood decreases rapidly for the first three days after transfusion and then remains constant. This indicates that after this time label is being lost from both sets of animals at about the same rate and implies that the cells remaining at this time are surviving normally. The ratio becomes constant at about 0.5 which indicates that the discrepancy between the two curves is caused by destruction of the 50% parasitized erythrocytes during the first three days.
C. Basophilic Erythrocyte

The survival of Cr$^{51}$ labeled basophilic erythrocytes, expressed as percent Cr$^{51}$ remaining at intervals over a period of one month is given in Table 7. The blood sample used for the survival study consisted almost entirely of young blood cells out of which about 80% were basophilic erythrocytes. If these young cells had a normal life span they should remain in the circulation until they would be destroyed at the end of their normal life span at around 54 days of age. To estimate the percent Cr$^{51}$ expected to remain on any given day ($N_t$) the formula $N_t = No e^{-kt}$ was used. This formula assumes no senescent loss but considers random losses such as elution.

Figure 24 shows the actual curve and the curve expected if the basophilic erythrocytes survived for a normal period and the only Cr$^{51}$ loss was by elution of label. The ratio of the two curves at intervals over a period of thirty days is also shown. A discrepancy between the expected and actual basophilic erythrocyte survival curves is apparent. This discrepancy indicates that the basophilic erythrocytes do not survive to the normal age of erythrocytes. The extent of basophilic erythrocyte destruction is remarkable, instead of surviving for the normal rat erythrocyte life span of 54 days, these cells are destroyed at a rate of about 2.5% per day which is slightly higher than the 1.8% per day destruction rate of normal blood cells.

Figure 25 shows a comparison of a survival curve of normal erythrocytes and basophilic erythrocytes from rats just free of parasites. During the first two days the basophilic erythrocytes are destroyed at a
Figure 24. Survival of basophilic erythrocytes (80% basophilia) transfused from a rat recovering from P. berghei infection to compatible drug treated non-infected rats. The basophilic erythrocyte survival curve was drawn with average data from three rats. The basophilic erythrocytes had a variety of life spans ranging from normal to a few days. As a result the curve generated by the labeled cohort was similar in form to a curve generated by a random label of normal erythrocytes.
observed to expected

% Cr\textsuperscript{51} actually remaining

% Cr\textsuperscript{51} expected to remain

ratio of observed to expected

FIGURE 24

DAYS

0 10 20 30 40 50 60 80 100

%
Figure 25. Survival curves of normal and post-infection basophilic erythrocytes. During the first two days after transfusion both normal and basophilic erythrocytes are destroyed at a similar rate. Thereafter destruction of basophilic erythrocytes decreases for a few days. Between four to ten days basophilic erythrocytes are destroyed at a faster rate than the normal erythrocytes but thereafter the basophilic and normal erythrocyte survival curves run parallel. This indicates that during this time label is being lost from both sets of animals at about the same rate and implies that the basophilic erythrocytes remaining at this time are surviving about as would a population of erythrocytes in an animal that had never been subjected to a severe bout of anemia.
rate almost equal to that of normal erythrocytes, thereafter the rate of
destruction decreases for a few days. Between four to ten days the baso-
philic erythrocytes are destroyed at a rate faster than the normal
erythrocytes. Finally the basophilic erythrocyte survival curve runs
parallel to the normal erythrocyte survival curve indicating that the
rate of destruction of the basophilic erythrocytes is similar to the
rate of destruction of erythrocytes in a population of erythrocytes from
an animal that never had an anemic crisis.

D. Post infection mature erythrocyte

Basophilic erythrocytes produced during the recovery period of
malarial infection show abnormal life spans. To study the survival of
post recovery mature erythrocytes a survival study was done using blood
collected from a rat fifteen days after peak parasitemia. At the time
of blood transfusion the level of basophilia and packed cell volume of
the donor rat was within normal range (Basophilia - 2%, P.C.V. - 46%).

The survival of post infection mature erythrocytes expressed as
$\% Cr^{51}$ remaining over a period of thirty days is shown in Figure 26.
Except for slight variations in the early part of the study period, the
post infection normal erythrocyte survival curve appears to resemble a
normal erythrocyte survival curve. From the shape of the curve it
appears that the erythrocytes produced during the later part of the
recovery period or surviving into this period behave like normal
erythrocytes.
Figure 26. Survival after transfusion into compatible non-infected rats of mature erythrocytes present in a rat fifteen days after the end of patent parasitemia. The curve was constructed from average data from five rats. The curve is similar to a normal erythrocyte survival curve. This indicates that the erythrocytes present at the time of labeling have a normal distribution of life expectancies. A surprising observation when it is realized that most of them were produced during a period of about one third of the life span of the normal rat erythrocyte.
% Cr\textsuperscript{51} remaining

DAYS

FIGURE 26
DISCUSSION

In spite of an enormous amount of work the pathophysiology of malaria is still unclear. For a long time the effects of direct invasion by the parasite on the host erythrocyte were considered to be the primary cause of pathology, but in recent years as widespread effects of the disease on all erythrocytes have been reported, workers in the field have proposed other mechanisms of pathology. Although the concept that plasmodial infection involves all erythrocytes is not new (Overman, 1948) only in recent years has this concept gained experimental substantiation. Zuckerman (1963) and several other workers (Devakul and Maegraith, 1959; Stohlman et al., 1963) have reported destruction of both parasitized as well as non-parasitized erythrocytes in animals with malaria; Danon (1962) and other workers have reported abnormally high osmotic fragility of both infected and non-infected cells in animals with malaria; Overman (1948) and more recently Dunn (1969) have shown that alterations occur in sodium and potassium transport by membranes of most of the blood cells during malarial infection. In addition to release of parasitic excretory products, cell debris, and enzymes there have been reported to be increases in the concentration of hemolytic (Laser, 1950) and toxic substances (Riley and Maegraith, 1961) in the circulations of animals with malaria. It is possible that non-infected erythrocytes may be damaged by contact with such products in the circulation of an animal with malaria.
Kreier (1969) suggested that the mechanism of erythrocyte destruction in malaria may be dependent on membrane damage due to both direct or indirect effects of the parasites. Arnold et al. (1969) in the same year reported that by scanning and transmission electron microscopy of Plasmodium infected human and mouse erythrocytes it was possible to observe large "holes" in the parasitized as well as parasite free blood cells. They indicated that these "holes" were caused by the direct or indirect action of the parasites. They also suggested that the "holes" in parasite free cells were caused by the movement of the parasites from an infected cell to another cell.

Our results suggest that erythrocyte abnormalities of various magnitudes occur at various stages of the infection. During early infection most of the blood cells remain completely normal, and even the parasitized blood cells do not show lesions suggestive of membrane damage at the time of parasite entry into the cell. Ladda et al. (1969) made a study of the mode of penetration of merozoites in mammalian and avian erythrocytes. They concluded that during the process of penetration, the continuity of the host cell membrane is not disrupted, rather the merozoite enters the erythrocytes by invagination of the membrane and that after completion of the entrance, the edges of the orifice fuse. The results of the present study are compatible with the conclusions of Ladda et al. (1969) or can be explained by the simpler hypothesis that the erythrocyte membrane is capable of healing itself after penetration by the merozoite.
The most common abnormality observable during the early stage of infection is the swollen nature and the distortion of some of the parasitized cells. Examination of the cells by various light and electron microscope techniques suggests that the location and size of the parasite inside the cell determines the magnitude and type of distortion which occurs. Some of the cells containing small parasites may remain completely normal in shape whereas others with larger parasites may show morphological abnormalities of magnitudes ranging from a slight swelling to sufficiently severe swelling to cause the erythrocyte to assume a signet ring shape.

As the level of parasitemia increases, blood cells with morphological abnormalities become more common. Scanning electron microscope examination of erythrocytes from mice with moderate levels of parasitemia, reveals that some of the infected cells do appear to have "holes" in their membranes, quite similar in nature to the "holes" reported by Arnold et al. (1969). These "holes" are never seen in erythrocytes from normal mice nor in non-infected erythrocytes in parasitized blood samples. When comparable infected blood cells are examined by the combined Giemsa staining light microscopy and carbon replica electron microscopy techniques, then the "holes" in the infected erythrocytes are not seen. Instead some of the erythrocytes containing large parasites have depressions or cavities of various sizes mostly in the region where the parasite is located. These depressions or cavities appear to be caused by a "sinking in" of the erythrocyte membrane. It is possible that by the scanning electron microscopy these depressions, which have intact membranes covering them, may appear to be "holes" because the bottom of
the depression did not get a sufficient gold coat during shadowing or because of the angle of examination the bottom of the depression could not be seen.

These observations raise the question of what causes the formation of these cavities or depressions exactly over the parasite. One answer to this question may be that ingestion of the host cytoplasm by the parasite or some toxic substance or enzyme causes localized alteration in the internal structure of the blood cells. It is also probable that the parasites are less dense than the erythrocytes and that during the process of fixation and drying they shrink more and thus depressions are formed in the erythrocyte over the parasite.

There are frequent and severe abnormalities in mouse erythrocytes collected during the stage of high parasitemia. The infected cells are large and twisted. Many are spherocytic and have granular surfaces. At the stage of peak parasitemia these abnormalities are not limited to the infected cells, as some of the unparasitized cells also have similar abnormalities. It appears that during the stage of peak parasitemia the concentration of parasite excretory products or cell debris is so great that even the non-infected cells are damaged by being in the circulation.

In mice the relationship between parasitization and change in erythrocyte morphology can thus be summarized as follows: After penetration of a merozoite into a cell, the membrane closes over the entry point leaving no detectable lesion. During the early stages of the parasites growth the infected blood cell may show no morphological abnormalities. But as the parasite becomes larger it consumes hemoglobin...
and damages the cell structure. The cell may become flattened except
where the parasite is located and thus may assume a signet ring form.
As the parasite further increases in size it consumes even more hemo-
globin and damages the internal structure of the erythrocyte to a
greater degree. The erythrocyte then becomes grossly distorted and
may show swelling in some regions and become shrunken in others. As
a result deep irregular folds and depressions are formed in the
erythrocyte membrane. Finally the erythrocyte loses its osmotic con-
trol completely, becomes spherocytic and ruptures. After rupture of
parasitized cells, a large quantity of parasite excretory products such
as malarial pigments, enzymes, and cell debris are released into the
circulation. As the parasitemia increases the concentration of these
products becomes high in the circulation and they may cause damage to
membranes of the non-parasitized cells. This may lead to the morpholo-
gical alteration of even the non-parasitized cells.

During early stages of P. berghei infection in rats, erythrocyte
abnormalities quite similar to those seen in erythrocytes of mice are
found. Erythrocytes containing small parasites show little or no
change, but as the parasites increase in size they cause swelling where
they are located which causes the erythrocytes to assume a signet ring
or oval shape.

As the parasitemia increases, frequency of erythrocytes with large
parasites increases. These erythrocytes show severe morphological
abnormalities. The cells assume a spherocytic form and cells containing
mature schizonts may develop irregular and rough membranes.
In infected rats even during the stage of peak parasitemia most of the morphological abnormalities occur in the infected erythrocytes and the uninfected erythrocytes retain their normal shape. In this latter respect the events in the infected mouse differ from those in the rat. This difference may be due in part to the fact that in mice the parasitemia may exceed 60 to 70% and thus vast amounts of parasite excretory products, and enzymes are released into the circulation. On the other hand rats generally start to recover after reaching 50 to 60% parasitemia and thereafter most of the foreign substances are probably rapidly removed from the circulation.

The results of our survival studies on *P. berghei* infected erythrocytes agree with our observations on morphological effects of infection. After transfusion of *P. berghei* infected rat erythrocytes with about 50% parasitemia into normal animals, only a portion of the transfused cells, approximately equal to the proportion parasitized are removed from the circulation at a rate greater than normal. The nonparasitized cells in the recipient animals appear to behave like normal erythrocytes.

Observations similar to ours have also been reported by other workers. George et al. (1966) reported a normal survival time of erythrocytes from infected donor rats after transfusion into normal animals. Kreier and Leste (1967) studied the survival of parasitized rat erythrocytes with various levels of parasitemia, ranging between 1 to 20%, after transfusion into normal rats. During their study after initial removal of parasitized erythrocytes the remaining erythrocytes appeared to survive like normal cells and thus there was no indication
that nonparasitized rat erythrocytes were damaged sufficiently to cause their premature destruction in normal animals.

In infected rats the most severe alterations in erythrocyte morphology are seen during the period of recovery. At that stage erythrocytes of large size and irregular form with pitted surfaces are seen in the circulation. These latter cells were identified by the use of combined Giemsa staining light microscopy and carbon replica electron microscopy techniques as immature basophilic erythrocytes or reticulocytes. It appears that most of the erythrocyte morphological abnormalities seen during the recovery period of *P. berghei* infection are the result of the production of immature basophilic erythrocytes during a period of anemic "stress" rather than being caused by the action of parasites or parasite products.

It was observed that abnormalities such as pitted surfaces and irregular form are found also in the basophilic erythrocytes produced following phenylhydrazine treatment or repeated cardiac bleeding as well as in animals with malaria. In our studies the degree of morphological alteration in basophilic erythrocytes produced following cardiac bleeding was less than that seen in reticulocytes produced during the recovery stage of the malarial infection. This may have been due to the fact that our plasmodium strain caused more severe anemia and a greater basophilia (eighty or ninety percent of the total blood cell population) than that produced by cardiac bleeding (twenty percent basophilic erythrocytes).
In the recovering rats the most severe morphological alteration occurred in reticulocytes produced immediately after removal of most of the parasitized cells from the circulation. These cells were large and flat and by phase contrast microscope examination appeared to be ring shaped. This particular appearance may be due to incomplete synthesis of hemoglobin in these cells. As an animal continues to recover cells of more normal size and shape are produced.

The presence in the animal of predominantly morphologically abnormal erythrocytes during the recovery raises the important question whether these cells mature and become normal erythrocytes or whether they remain abnormal and are removed from the circulation prematurely. To answer this question we determined the survival of reticulocytes produced by rats during the period of recovery from infection. The survival studies were done by transfusing Cr\(^{51}\) labeled blood into normal animals, to avoid the complications on interpretation of the internal environment of a malaria infected or recently recovered animal.

The blood sample used for the survival studies was collected from a rat at the stage of infection immediately after the disappearance of parasitemia. The blood contained almost all young blood cells out of which about eighty percent were basophilic erythrocytes. As rat erythrocytes have an average life span of about fifty-four days, basophilic erythrocytes produced under normal conditions remain in the circulation until they reach about fifty-four days of age and are then destroyed due to senescence. But the reticulocytes examined during the present studies do not have normal life spans. Instead of living for about
fifty-four days half of them were destroyed within two weeks and by the end of the one month study period only ten percent of them remained in the circulation.

Possession of an abnormal life span is not unique to the reticulocytes produced following malarial infection. It has been reported by other workers (Berlin and Lotz, 1951; Stohlman, 1961; Card and Valverg, 1967) that the reticulocytes produced following severe bleeding or during an anemic stress caused by a variety of diseases, also have short life spans.

Premature destruction of "stress" reticulocytes may have a variety of causes. During the period of anemic stress the reticulocytes are produced at a rapid rate and are released into the circulation prematurely. It is possible that these cells may lack something essential for the normal function of erythrocytes and therefore they remain physiologically unsuitable to survive. For example Card et al. (1969) reported that the macrocytic reticulocytes produced in rabbits in response to cardiac bleeding disappear from the circulation at two rates one group has a mean life span of 3.1 days and the other 28 days. Normal rabbit erythrocytes have a mean life span of 66 days. They also reported that during glucose deprivation autohemolysis of macrocytic reticulocytes was significantly greater than that of mature erythrocytes.

It is a well known fact that spleen, liver, bone-marrow, etc. selectively trap abnormal cells and remove them from the circulation. The trapping works effectively against cells having increased thickness and greater agglutinability such as occurs with antibody coating.
Immature reticulocytes being both bigger and more agglutinable because of transferrin coating are doubly vulnerable to sequestration.

Sorbie and Valberg (1970) studied splenic sequestration of stress erythrocytes and indicated that the spleen selectively sequesters stress macrocytic reticulocytes and retains them for much longer periods than the normal reticulocytes.

If we consider the overall process of recovery from severe anemic stress then production of erythrocytes with various life spans appears beneficial. For example during the period of recovery from malarial infection rats replace eighty to ninety percent of the blood cell population by newly formed basophilic erythrocytes within a few days. If all these cells produced had normal life spans then after a period of about fifty days most of these cells should again be destroyed within a few days leading to another anemic bout. On the other hand if an animal recovering from anemia produces erythrocytes with various life-spans then the erythrocyte population is replaced by new blood cells over a period of time and thus the animal adjusts itself physiologically to the normal pattern of erythrocyte sequestration and replacement.

As a matter of fact our studies of survival of erythrocytes of animals in the post recovery stages of the malarial infection clearly indicate production of erythrocytes of varying ages. Although most of the cells produced during the earlier part of the recovery period are very short lived, nevertheless some cells of longer life span are also produced. As the animal continues to recover, the short lived cells are progressively removed and replacement erythrocytes of longer more nearly
normal life spans are produced. Our erythrocyte survival studies on blood collected two weeks after the peak parasitemia yielded a survival curve which closely resembled a normal erythrocyte survival curve. This indicates that within two weeks after peak parasitemia most of the very short lived reticulocytes were removed from the circulation and that the replacement erythrocytes had a distribution of life spans which approximated the life expectancies of erythrocytes in a population of normal erythrocytes.

Erythrocyte destruction not related to direct parasite action during the recovery period of malarial infection has been reported by various workers. It is surprising that no one has studied the behavior of "stress" reticulocytes during this period. Common hypotheses explaining the excessive post-infection erythrocyte destruction deal mostly with postulated effects on hypersplenism or with autoimmune mechanisms.

There is no concrete evidence that an autoimmune mechanism is involved in destruction of erythrocytes in animals with malaria. Most reports on studies of autoantibody in malarious animals merely report the occurrence of autoantibody (McGhee, 1964; Sloan and McGhee, 1965; Kreier et al., 1966; etc.). Zuckerman (1960) detected antibody coating of blood cells during malarial infection by direct Coomb's tests, but later she questioned the validity of her findings when she noticed that positive Coomb's reactions due to the presence of reticulocytes occurred in animals with artificially induced anemias. Similarly George et al. (1960) were unable to find positive Coomb's reactions in *P. berghei* infected rats when they used rabbit serum specific for immuno-gamma globulin as reagent.
Kreier and Leste (1968) treated normal and infected rat erythrocytes with globulin from *P. berghei* infected rats and transfused the cells to normal rats along with the infected rat globulin. They did not observe any increase in the degree of destruction of parasitized or nonparasitized erythrocytes as result of treatment with infected rat globulin. A similar result was reported by Kreier (1969) in regard to the effect of infected chicken serum on the survival of normal chicken erythrocytes. He did not notice accelerated destruction of normal erythrocytes incubated in infected chicken serum before transfusion, nor did injection of infected chicken serum into normal chickens cause erythrocyte destruction.

In light of the present observations regarding morphological abnormalities and production of short lived erythrocytes after malarial infection, it appears that most non-infected erythrocytes destroyed during the recovery period are "stress" reticulocytes. The present studies were conducted by transfusing "stress" reticulocytes into normal animals. So the destruction of those cells was not due to the involvement of an autoimmune mechanism nor to hypersplenism nor involvement of hemolytic substances. It is possible that the presence of hypersplenism and other abnormal conditions in an infected animal might cause very rapid destruction of "stress" reticulocytes. A comparative study dealing with survival of "stress" reticulocytes in normal animals and one recovering from malaria may answer the above question.

The destruction of short lived erythrocytes may account for most erythrocyte destruction in animals in the recovery period of malarial
infection. The cause of destruction of nonparasitized erythrocytes during the period of parasitemia (at least in certain species of animals) still remains an unanswered question. Whether this is due to the action of hemolytic factors as suggested by Laser (1950) or to the action of toxic substances as suggested by Riley and Maegraith (1961) or to physical damage of erythrocytes during an attempt by the merozoites to enter the cells as suggested by Kreier (1971) is unsettled.

Our morphological studies of rat and mouse erythrocytes did not reveal any evidence that non-infected erythrocytes were damaged during the early stages of the infection. If non-infected erythrocytes are damaged due to one or more of the causes proposed by various authors then probably either they were destroyed promptly or trapped by the internal organs so that they did not remain in the circulation long enough to be detected in our morphological or survival studies.
SUMMARY AND CONCLUSIONS

Mouse and rat erythrocytes collected from animals in various stages of *P. berghei* infection were examined by a number of light and electron microscope techniques i.e., light microscopy of Giemsa stained erythrocytes, phase contrast and Nomarski interference microscopy of fresh blood, light microscopy of fixed blood cells after Giemsa staining and carbon shadowing, electron microscopy of carbon replicas of erythrocytes and scanning electron microscopy of gold coated blood cells. The electron microscopy was done in conjunction with a photomicroscopy technique by which it was possible to identify individual infected, non-infected, and basophilic erythrocytes seen by the electron microscope.

Studies of infected erythrocytes by various microscopic techniques revealed that penetration by a merozoite produces little or no effect on erythrocytes of mice or rats. When there is a low parasitemia, most of the infected blood cells containing small parasites appear normal and maintain regular, biconcave disk shapes. Blood cells which contain larger parasites appear swollen or signet ring shaped at this stage of the infection.

As the parasites grow inside the blood cells they consume hemoglobin and damage the internal structure of the erythrocyte. This causes morphological change. The infected erythrocytes become distorted and may appear swollen or shrunken in the region where the parasite is
located. With increases in parasitemia the proportion of erythrocytes showing abnormalities also increases.

In mice, with high parasitemia, many irregularly shaped erythrocytes are found. Some have deep irregular folds and depressions in their membranes, others may be spherocytic and may have granular surfaces. These abnormalities are not limited to the infected cells, as some of the non-infected erythrocytes also appear abnormal.

In rats even during the stage of peak parasitemia (50%) most of the morphological abnormalities occur in the infected erythrocytes. Survival studies on fifty percent infected rat erythrocytes transfused into normal rats substantiated the morphological observations because only the parasitized erythrocytes were rapidly destroyed. This suggests that in rats even during the stage of peak parasitemia most of the cell damage is confined to the infected cells and the non-infected erythrocytes are not damaged to an extent to become morphologically or physiologically abnormal.

During the recovery stage in rats a large number of basophilic erythrocytes are produced. These erythrocytes are distorted and have pitted surface membranes. Some of the membrane characteristics and irregularities are also found in basophilic erythrocytes produced in response to blood loss following bleeding or phenylhydrazine treatment. Survival studies on post-recovery basophilic erythrocytes transfused into normal animals indicated that during the early part of the recovery period erythrocytes of various life-spans are produced. Many of the erythrocytes with short life-spans are removed from the circulation
within a few days and are replaced by erythrocytes of longer life-span. It appears that destruction of short lived basophilic erythrocytes might be responsible for many reported observations of destruction of non-parasitized erythrocytes in animals in the recovery stage of the malarial infection.
APPENDIX I

Formulas for Solutions

1. **Phosphate Buffer (PB), pH 7.2**
   - Potassium phosphate, monobasic \((\text{KH}_2\text{PO}_4)\) ........ 7.7 gm.
   - Sodium phosphate, dibasic anhydrous \((\text{Na}_2\text{HPO}_4)\) ... 20.3 gm.
   - Double Distilled water .................. 3 liters

2. **Phosphate Buffered Saline (PBS), pH 7.2**
   - Potassium phosphate, monobasic \((\text{KH}_2\text{PO}_4)\) ........ 7.7 gm.
   - Sodium phosphate, dibasic anhydrous \((\text{Na}_2\text{HPO}_4)\) ... 20.3 gm.
   - Sodium chloride ......................... 25.5 gm.
   - Double Distilled water .................. 3 liters

3. **Organic Solvent Solution**
   - Potassium permanganate .................. 0.15 gm.
   - Potassium dichromate ...................... 0.15 gm.
   - Concentrated sulphuric acid .............. 1.5 ml.
### APPENDIX II

#### Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Course of <em>P. berghei</em> infection in adult mice after intra-peritoneal injection of $2 \times 10^6$ parasitized erythrocytes</td>
<td>129</td>
</tr>
<tr>
<td>2. Course of <em>P. berghei</em> infection in adult rats after intra-peritoneal injection of $2 \times 10^6$ parasitized erythrocytes</td>
<td>131</td>
</tr>
<tr>
<td>3. Effect of phenylhydrazine treatment on the production of basophilic erythrocytes in an adult rat</td>
<td>133</td>
</tr>
<tr>
<td>4. Effect of cardiac bleeding on the production of basophilic erythrocytes in an adult rat</td>
<td>134</td>
</tr>
<tr>
<td>5. Survival of Cr$^{51}$ labeled normal rat erythrocytes after transfusion into homologous normal rats</td>
<td>135</td>
</tr>
<tr>
<td>6. Survival of Cr$^{51}$ labeled infected erythrocytes (50% parasitemia) after transfusion into homologous normal rats</td>
<td>137</td>
</tr>
<tr>
<td>7. Survival of Cr$^{51}$ labeled post infection basophilic erythrocytes after transfusion into homologous normal rats</td>
<td>139</td>
</tr>
<tr>
<td>8. Survival of Cr$^{51}$ labeled post infection mature erythrocytes after transfusion into homologous normal rats</td>
<td>141</td>
</tr>
</tbody>
</table>
Table 1

COURSE OF *P. BERGHEI* INFECTION IN ADULT MICE AFTER INTRAPERITONEAL INJECTION OF $2 \times 10^6$ PARASITIZED ERYTHROCYTES

<table>
<thead>
<tr>
<th>Days After Infection</th>
<th>Mouse #1</th>
<th>Mouse #2</th>
<th>Mouse #3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Para $^1$</td>
<td>Baso $^2$</td>
<td>PCV $^3$</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>4</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td>5</td>
<td>76</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>Died</td>
<td>Died</td>
<td>Died</td>
</tr>
</tbody>
</table>
Table 1 (continued)

COURSE OF P. BERGHEI INFECTION IN ADULT MICE AFTER INTRAPERITONEAL INJECTION OF $2 \times 10^6$ PARASITIZED ERYTHROCYTES

<table>
<thead>
<tr>
<th>Days After Infection</th>
<th>Mouse #4</th>
<th></th>
<th></th>
<th>Mouse #5</th>
<th></th>
<th></th>
<th>Average</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Para</td>
<td>Baso</td>
<td>PCV.</td>
<td>Para</td>
<td>Baso</td>
<td>PCV.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>1</td>
<td>2</td>
<td>49</td>
<td>0</td>
<td>2</td>
<td>52</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3</td>
<td>4</td>
<td>49</td>
<td>2</td>
<td>1</td>
<td>52</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>15</td>
<td>2</td>
<td>46</td>
<td>8</td>
<td>2</td>
<td>50</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>37</td>
<td>5</td>
<td>37</td>
<td>28</td>
<td>3</td>
<td>46</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>69</td>
<td>3</td>
<td>22</td>
<td>52</td>
<td>6</td>
<td>28</td>
<td>67</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>96</td>
<td>7</td>
<td>9</td>
<td>84</td>
<td>12</td>
<td>11</td>
<td>90</td>
<td>9</td>
</tr>
</tbody>
</table>

1 Para = % Parasitemia
2 Baso = % Basophilia
3 PCV = % Packed Cell Volume
### Table 2

**COURSE OF *P. BERGHEI* INFECTION IN ADULT RATS AFTER INTRAEPITONEAL INJECTION OF $2 \times 10^6$ PARASitized ERYTHROCYTES**

| Days After Infection | Rat #1 | | | | Rat #2 | | | | Rat #3 | | |
|----------------------|--------|---|---|---|--------|---|---|---|--------|---|---|---|
|                      | TP$_1$ | BP$_2$ | B$_3$ | PCV$_4$ | TP | BP | B | PCV | TP | BP | B | PCV |
| 1                    | 0      | 0    | 1    | 50     | 0   | 0   | 2  | 52  | 0   | 0   | 3  | 48  |
| 2                    | 0      | 0    | 3    | 48     | 0   | 0   | 2  | 51  | 0   | 0   | 4  | 50  |
| 3                    | 3      | 0    | 2    | 49     | 1   | 0   | 1  | 48  | 0   | 0   | 2  | 49  |
| 4                    | 8      | 2    | 1    | 44     | 4   | 1   | 1  | 47  | 2   | 0   | 2  | 47  |
| 5                    | 17     | 4    | 0    | 40     | 8   | 2   | 0  | 45  | 5   | 2   | 0  | 46  |
| 6                    | 30     | 3    | 0    | 27     | 16  | 2   | 0  | 40  | 13  | 3   | 0  | 41  |
| 7                    | 51     | 8    | 0    | 20     | 33  | 3   | 0  | 36  | 25  | 2   | 0  | 37  |
| 8                    | 62     | 20   | 6    | 16     | 57  | 10  | 0  | 30  | 49  | 8   | 0  | 29  |
| 9                    | 40     | 38   | 16   | 22     | 74  | 30  | 2  | 20  | 64  | 31  | 4  | 18  |
| 10                   | 30     | 29   | 46   | 30     | 65  | 57  | 12 | 14  | 59  | 56  | 20 | 11  |
| 11                   | 9      | 9    | 60   | 36     | 45  | 43  | 40 | 17  | 49  | 47  | 42 | 18  |
| 12                   | 0      | 0    | 90   | 40     | 18  | 12  | 80 | 22  | 22  | 18  | 76 | 24  |
| 13                   | 0      | 0    | 48   | 43     | 0   | 0   | 60 | 30  | 0   | 0   | 62 | 33  |
| 14                   | 0      | 0    | 30   | 45     | 0   | 0   | 36 | 37  | 0   | 0   | 31 | 40  |
Table 2 (continued)

COURSE OF *P. BERGHEI* INFECTION IN ADULT RATS AFTER INTRAPERITONEAL INJECTION OF $2 \times 10^6$ PARASITIZED ERYTHROCYTES

<table>
<thead>
<tr>
<th>Days After Infection</th>
<th>Rat #4</th>
<th>Rat #5</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TP</td>
<td>BP</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>55</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>72</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>67</td>
<td>62</td>
<td>14</td>
</tr>
<tr>
<td>11</td>
<td>42</td>
<td>41</td>
<td>39</td>
</tr>
<tr>
<td>12</td>
<td>14</td>
<td>14</td>
<td>82</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>57</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

1. $^1TP = \%$ Total Parasitemia
2. $^2BP = \%$ Parasitized Basophilic Erythrocytes
3. $^3B = \%$ Non-Parasitized Basophilic Erythrocytes
4. $^4PCV = \%$ Packed Cell Volume
Table 3

EFFECT OF PHENYLHYDRAZINE TREATMENT ON THE PRODUCTION OF BASOPHILIC ERYTHROCYTES IN AN ADULT RAT (270 gms)

<table>
<thead>
<tr>
<th>Days</th>
<th>Drug</th>
<th>% Basophilia</th>
<th>% PCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8 mg.</td>
<td>4</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>8 mg.</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>--</td>
<td>25</td>
<td>34</td>
</tr>
<tr>
<td>Days</td>
<td>Bleeding</td>
<td>% Basophilia</td>
<td>% PCV</td>
</tr>
<tr>
<td>------</td>
<td>----------</td>
<td>--------------</td>
<td>--------</td>
</tr>
<tr>
<td>1</td>
<td>3 ml.</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>3 ml.</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>3 ml.</td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>2 ml.</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Days After Transfusion</td>
<td>Rat #1 CPM*/0.1 gm Blood</td>
<td>% Cr\textsuperscript{51} Remaining</td>
<td>Rat #2 CPM*/0.1 gm Blood</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------------------------</td>
<td>---------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>0</td>
<td>656</td>
<td>100</td>
<td>594</td>
</tr>
<tr>
<td>1</td>
<td>532</td>
<td>81</td>
<td>493</td>
</tr>
<tr>
<td>3</td>
<td>485</td>
<td>74</td>
<td>439</td>
</tr>
<tr>
<td>5</td>
<td>394</td>
<td>60</td>
<td>314</td>
</tr>
<tr>
<td>7</td>
<td>338</td>
<td>51</td>
<td>257</td>
</tr>
<tr>
<td>9</td>
<td>288</td>
<td>44</td>
<td>206</td>
</tr>
<tr>
<td>11</td>
<td>249</td>
<td>37</td>
<td>188</td>
</tr>
<tr>
<td>14</td>
<td>185</td>
<td>28</td>
<td>154</td>
</tr>
<tr>
<td>18</td>
<td>146</td>
<td>22</td>
<td>118</td>
</tr>
<tr>
<td>20</td>
<td>119</td>
<td>18</td>
<td>102</td>
</tr>
</tbody>
</table>
Table 5 (continued)

SURVIVAL OF Cr^{51} LABELED NORMAL RAT ERYTHROCYTES AFTER TRANSFUSION INTO HOMOLOGOUS NORMAL RATS.

<table>
<thead>
<tr>
<th>Days After Transfusion</th>
<th>Rat #4</th>
<th>CPM/0.1 gm Blood</th>
<th>% Cr^{51} Remaining</th>
<th>Average Cr^{51} Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>1482</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>1310</td>
<td>88</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>982</td>
<td>66</td>
<td>74</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>902</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>693</td>
<td>46</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>633</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>500</td>
<td>33</td>
<td>35</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>399</td>
<td>26</td>
<td>29</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>293</td>
<td>19</td>
<td>21.5</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>305</td>
<td>20</td>
<td>19</td>
</tr>
</tbody>
</table>

*CPM = Count per minute
### Table 6

**SURVIVAL OF Cr\textsuperscript{51} LABELED INFECTED ERYTHROCYTES (50\% PARASITEMIA) AFTER TRANSFUSION INTO HOMOLOGOUS NORMAL RATS**

<table>
<thead>
<tr>
<th>Days After Transfusion</th>
<th>Rat #1</th>
<th>Rat #2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPM/0.1 gm Blood</td>
<td>% Cr\textsuperscript{51} Remaining</td>
</tr>
<tr>
<td>0</td>
<td>832</td>
<td>100</td>
</tr>
<tr>
<td>1/2</td>
<td>784</td>
<td>94</td>
</tr>
<tr>
<td>1 1/2</td>
<td>472</td>
<td>57</td>
</tr>
<tr>
<td>2 1/2</td>
<td>448</td>
<td>54</td>
</tr>
<tr>
<td>3 1/2</td>
<td>347</td>
<td>42</td>
</tr>
<tr>
<td>5 1/2</td>
<td>313</td>
<td>36</td>
</tr>
<tr>
<td>7 1/2</td>
<td>267</td>
<td>32</td>
</tr>
</tbody>
</table>
### Table 6 (continued)

**SURVIVAL OF Cr$^{51}$ LABELED INFECTED ERYTHROCYTES (50% PARASITEMIA) AFTER TRANSFUSION INTO HOMOLOGOUS NORMAL RATS**

<table>
<thead>
<tr>
<th>Days After Transfusion</th>
<th>CPM/0.1 gm Blood</th>
<th>% Cr$^{51}$ Remaining</th>
<th>Average Cr$^{51}$ Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1012</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1/2</td>
<td>941</td>
<td>93</td>
<td>94</td>
</tr>
<tr>
<td>1 1/2</td>
<td>582</td>
<td>57</td>
<td>56.5</td>
</tr>
<tr>
<td>2 1/2</td>
<td>521</td>
<td>52</td>
<td>49</td>
</tr>
<tr>
<td>3 1/2</td>
<td>398</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>5 1/2</td>
<td>334</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>7 1/2</td>
<td>263</td>
<td>26</td>
<td>27</td>
</tr>
</tbody>
</table>

*CPM = Count per minute*
Table 7

SURVIVAL OF Cr\(^{51}\) LABELED POST INFECTION BASOPHILIC ERYTHROCYTES
AFTER TRANSFUSION INTO HOMOLOGOUS NORMAL RATS

<table>
<thead>
<tr>
<th>Days After Transfusion</th>
<th>Rat #1 CPM(^*)/0.1 gm Blood</th>
<th>% Cr(^{51}) Remaining</th>
<th>Rat #2 CPM/0.1 gm Blood</th>
<th>% Cr(^{51}) Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>448</td>
<td>100</td>
<td>573</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>407</td>
<td>91</td>
<td>452</td>
<td>79</td>
</tr>
<tr>
<td>2</td>
<td>387</td>
<td>86</td>
<td>430</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>339</td>
<td>76</td>
<td>423</td>
<td>74</td>
</tr>
<tr>
<td>6</td>
<td>334</td>
<td>75</td>
<td>372</td>
<td>65</td>
</tr>
<tr>
<td>8</td>
<td>250</td>
<td>56</td>
<td>286</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>221</td>
<td>44</td>
<td>157</td>
<td>27</td>
</tr>
<tr>
<td>12</td>
<td>175</td>
<td>39</td>
<td>70</td>
<td>12</td>
</tr>
<tr>
<td>14</td>
<td>124</td>
<td>28</td>
<td>64</td>
<td>11</td>
</tr>
<tr>
<td>16</td>
<td>92</td>
<td>21</td>
<td>56</td>
<td>10</td>
</tr>
<tr>
<td>18</td>
<td>90</td>
<td>20</td>
<td>48</td>
<td>8</td>
</tr>
<tr>
<td>20</td>
<td>59</td>
<td>13</td>
<td>38</td>
<td>7</td>
</tr>
<tr>
<td>22</td>
<td>48</td>
<td>11</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>26</td>
<td>45</td>
<td>10</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>45</td>
<td>10</td>
<td>24</td>
<td>4</td>
</tr>
</tbody>
</table>
### Table 7 (continued)

**SURVIVAL OF Cr$^{51}$ Labeled Post Infection Basophilic Erythrocytes**

*After Transfusion into Homologous Normal Rats:*

<table>
<thead>
<tr>
<th>Days After Transfusion</th>
<th>Rat #3</th>
<th>% Cr$^{51}$ Remaining</th>
<th>Average Cr$^{51}$ Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPM/0.1 gm Blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>671</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>545</td>
<td>81</td>
<td>84</td>
</tr>
<tr>
<td>2</td>
<td>470</td>
<td>70</td>
<td>77</td>
</tr>
<tr>
<td>4</td>
<td>440</td>
<td>65</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>417</td>
<td>62</td>
<td>67</td>
</tr>
<tr>
<td>8</td>
<td>381</td>
<td>56</td>
<td>54</td>
</tr>
<tr>
<td>10</td>
<td>218</td>
<td>32</td>
<td>34</td>
</tr>
<tr>
<td>12</td>
<td>185</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>14</td>
<td>179</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>16</td>
<td>165</td>
<td>24</td>
<td>19</td>
</tr>
<tr>
<td>18</td>
<td>130</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>20</td>
<td>112</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>22</td>
<td>107</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>26</td>
<td>35</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

*CPM - Count per minute*
Table 8

SURVIVAL OF Cr\(^{51}\) Labeled Post Infection Mature Erythrocytes

After Transfusion Into Homologous Normal Rats

<table>
<thead>
<tr>
<th>Days After Transfusion</th>
<th>Rat #1</th>
<th>Rat #2</th>
<th>Rat #3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPM*/0.1 gm</td>
<td>% Cr(^{51}) Remaining</td>
<td>CPM/0.1 gm</td>
</tr>
<tr>
<td>0</td>
<td>535</td>
<td>100</td>
<td>822</td>
</tr>
<tr>
<td>1</td>
<td>534</td>
<td>100</td>
<td>825</td>
</tr>
<tr>
<td>3</td>
<td>568</td>
<td>106</td>
<td>714</td>
</tr>
<tr>
<td>5</td>
<td>496</td>
<td>93</td>
<td>838</td>
</tr>
<tr>
<td>8</td>
<td>336</td>
<td>63</td>
<td>357</td>
</tr>
<tr>
<td>11</td>
<td>282</td>
<td>53</td>
<td>301</td>
</tr>
<tr>
<td>14</td>
<td>211</td>
<td>40</td>
<td>248</td>
</tr>
<tr>
<td>17</td>
<td>149</td>
<td>28</td>
<td>208</td>
</tr>
<tr>
<td>20</td>
<td>129</td>
<td>24</td>
<td>202</td>
</tr>
<tr>
<td>24</td>
<td>105</td>
<td>20</td>
<td>145</td>
</tr>
<tr>
<td>27</td>
<td>84</td>
<td>16</td>
<td>126</td>
</tr>
<tr>
<td>30</td>
<td>59</td>
<td>11</td>
<td>90</td>
</tr>
</tbody>
</table>
Table 8 (continued)

SURVIVAL OF Cr\(^{51}\) LABELED POST INFECTION MATURE ERYTHROCYTES

AFTER TRANSFUSION INTO HOMOLOGOUS NORMAL RATS

<table>
<thead>
<tr>
<th>Days After Transfusion</th>
<th>Rat #4 CPM/0.1 gm Blood</th>
<th>5Cr(^{51}) Remaining</th>
<th>Rat #5 CPM/0.1 gm Blood</th>
<th>% Cr(^{51}) Remaining</th>
<th>Average Cr(^{51}) Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1134</td>
<td>100</td>
<td>1226</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>926</td>
<td>82</td>
<td>986</td>
<td>80</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>750</td>
<td>66</td>
<td>948</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>5</td>
<td>691</td>
<td>61</td>
<td>808</td>
<td>65</td>
<td>73</td>
</tr>
<tr>
<td>8</td>
<td>329</td>
<td>29</td>
<td>468</td>
<td>38</td>
<td>40</td>
</tr>
<tr>
<td>11</td>
<td>225</td>
<td>20</td>
<td>354</td>
<td>28</td>
<td>31</td>
</tr>
<tr>
<td>14</td>
<td>154</td>
<td>14</td>
<td>303</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>17</td>
<td>137</td>
<td>12</td>
<td>182</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>20</td>
<td>122</td>
<td>11</td>
<td>159</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>24</td>
<td>94</td>
<td>8</td>
<td>135</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>27</td>
<td>37</td>
<td>3</td>
<td>92</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>30</td>
<td>39</td>
<td>3</td>
<td>82</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^a\)CPM = Count per minute
LITERATURE CITED


temia and increased osmotic fragility of erythrocytes in rodent

Danon, D., Marikovsky, Y., and Gasko, O. 1966. Chromium$^{51}$ uptake as

Devakul, K., Maegraith, B. G. 1959. Lysis and others circulatory
Parasitol. 53:430-450.

Dodd, M. C., Wright, C., Baxter, J. A., Bouronade, B. A., Brunner, A. E.,
Winn, H. J. 1953. The immunologic specificity of antiserum for
trypsin-treated red blood cells and its reactions with normal and
hemolytic anemia cells. Blood. 8:640-647.

Donohue, D. M., Motulsky, A. G., Giblett, E. R., Pirziobirol, G.,
Viranuvalli, V., and Finch, C. A. 1955. The use of chromium as

Dunn, M. J. 1969. Alterations of red blood cell metabolism in simian
malaria: Evidence for abnormalities of non-parasitized cells.

Eaton, M. D. 1939. The soluble malarial antigen in the serum of monkeys

Ebaugh, F. G., Jr., Emerson, C. P., and Ross, J. F. 1953. The use of
radioactive chromium$^{51}$ as an erythrocyte tagging agent for the
determination of red cell survival in vivo. J. Clin. Invest. 32:
1260-1276.

Fogel, B. J., Shields, C. E., von Doenhoff, A. E., Jr. 1966. The
osmotic fragility of erythrocytes in experimental malaria. Am.

Fogel, B. J., von Doenhoff, A. E., Jr., Cooper, N. R., Fife, E. H., Jr.
1966. Complement in acute experimental malaria: I. Total hemo­

on erythrocytes of chickens infected with Plasmodium gallinaceum.

Geiman, Q. M., Antfinsen, C. B., McKee, R. W., Ormsbee, R. A., and Ball,
E.G. 1946. Studies on malarial parasites: VI. Methods and


