ABSTRACT

IMMUNOLOGIC AND PATHOLOGIC STUDIES OF INFECTIONS WITH RAT-ADAPTED *PLASMODIUM CHABAUDI*

By

Antony J. Musoke

Progress in the adaptation of *Plasmodium chabaudi* from the mouse to the rat host was indicated by progressive shortening of the prepatent period of infection, increases in rate of parasite growth, and lengthening of the period of patent parasitemia with successive passages in the new host. The data suggested that the parasite recently taken from the mouse host was more antigenic for rats than were the parasites taken from rats at subsequent passages. Another change noted was that *P. chabaudi* in rats did not produce the antigen responsible for acquired resistance to *Babesia rodhaini* as had been noted in infections of mice.

In rats, *P. chabaudi* produced moderate to severe anemia with splenomegaly and intravascular hemolysis, and glomerulonephritis with hemoglobinuria and proteinuria. However, the absence of high morbidity and mortality indicated that the disease was substantially less severe than that observed in mice. Disease signs in rats were associated with the presence of cold active agglutinins for trypsinized erythrocytes, the presence of soluble parasite antigen and concurrently, the presence of antibody to parasite antigen, in the plasma of diseased rats. Serologic studies indicated that in addition to their presence in blood serum, the parasite antigen and its antibody were present in homogenates of

kidney tissues and in the urine of acutely malarious rats. The ease with which the antigen and its antibody could be separated indicated that the antibody had a low avidity for its antigen.

It is suggested that these antigens and their low avidity antibodies form complexes that will react with erythrocytes causing them to be sequestered in the spleen and to be phagocytized. These complexes bound to red cells may also fix complement to cause the intravascular lysis of erythrocytes. Further, these complexes of antigen, antibody and complement could affect endothelial permeability and may act as immunotoxins. Thus they could be causal in the leakage of blood substances found in the kidneys and in the urine of rats with nephritis.

Since the complexes seem to react indiscriminately with and destroy infected as well as uninfected erythrocytes, this reaction, when insufficient to kill, may be instrumental in removing parasites from the blood and in the recovery from acute infection.

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# INTRODUCTION

Prior to the discovery of the rodent plasmodial parasite, *Plasmodium berghei* by Vincke and Lips (1948), investigations of malarial infections had been limited primarily to avian parasites such as *Plasmodium gallinaceum* (Brumpt, 1935) or *Plasmodium lophurae* (Coggishall, 1938) in domestic chickens or ducks. *P. berghei*, which was first detected in the African tree rat, *Thallomys spp.*, was found to be readily adaptable to laboratory mice and rats and quickly achieved popularity as a model for experimental mammalian malaria. In the meantime, further investigations of *Thallomys spp.* revealed that this rodent was a rich source of hemosporidian parasites. In addition to two new species of plasmodial parasites, *Plasmodium vinckei* (Vincke, 1948) and *Plasmodium chabaudi* (Landau, 1965), these animals furnished investigators the babesial parasite, *Babesia rodhaini*, as a model infection in laboratory rodents.

While *P. chabaudi* has not achieved the popularity of *P. berghei* as a research model for malaria, it seems in several respects to have traits that make it a desirable model for study. *P. chabaudi* is a parasite of normal erythrocytes and has a synchronized schizogony cycle. *P. berghei* infection, on the other hand, is asynchronous and requires immature erythrocytes as host cells. Protective immune responses could be induced in mice with *P. berghei* infection, but were variable and recrudescences of parasitemia were frequent with fatal relapses occurring as late as 4 months after development of latency (Cox, 1957;

1958). *P. chabaudi* produced stable immunization in infection and, in addition, stimulated the autoimmune-like responses that are associated with acute plasmodial and babesial infections (Cox and Milar, 1968; Cox *et al.*, 1966; Schroeder *et al.*, 1966; Sibinovic *et al.*, 1967a; 1967b; Corwin and Cox, 1969).

In research directed at defining the roles of specific parasite antigens and autoimmune or idiopathic immune substances in the pathogenesis and the acquired resistance of malaria, there has been a need for a plasmodial infection in mammals larger than mice that would furnish consistent results and the larger volumes of blood required. The mouse strain of *B. rodhaini* had proven to be readily adaptable to rats and has served as a basic model for immunopathologic studies of red cell infections (Schroeder *et al.*, 1966; Sibinovic *et al.*, 1967a; 1967b; 1969; Cox and Milar, 1968; Cox *et al.*, 1968; Corwin and Cox, 1969; Iturri and Cox, 1969). With the hope that *P. chabaudi* infection of rats might prove to be a suitable model, we adapted this parasite to rats and have studied the immunopathologic responses it produces in the new host.

# LITERATURE REVIEW

## *Plasmodium chabaudi*

### Origin and Taxonomic Status

*P. chabaudi* was isolated from *Thallomys rutilans*, a tree rat residing in the tropical rain forest at Bukoko, in the Central African Republic, by Landau (1965) and was discovered to have a high infection rate in adult *Thallomys* (Landau and Chabaud, 1966). A search for the natural vector of *P. chabaudi* by French investigators in 1965 was unsuccessful. However, *Anopheles stephensi* is an excellent laboratory host. Oocysts grow in the mid-gut, reaching a size of 50-80 μ, and sporozoites begin to appear in the salivary glands on the eleventh day after infection (Garnham, 1968).

*P. chabaudi* and *P. traguli* provide examples of mammalian species of *Plasmodium* in which exoerythrocytic stages have been detected in naturally infected hosts (Garnham, 1968). *P. chabaudi* develops preferentially in mature erythrocytes, though if reticulocytes are present in large numbers as after a crisis, the immature cells may occasionally be invaded. The blood picture of *P. chabaudi* infections shows slight differences according to the host, and the various species of rodents which are susceptible react in distinctive ways to the infection.

Unlike both *P. vinckei* and *P. berghei*, *P. chabaudi* causes reddening of mouse erythrocytes and differs from the former species in its erythrocytic forms, especially in the fragmentation of the nuclear

chromatin in young trophozoites (Granham, 1968).

Serologic and immunologic studies failed to establish antigenic differences between *P. chabaudi* and *P. vinckei* (Bray and El-Nahel, 1966; Spira and Zuckerman, 1966). The pattern of infection with *P. chabaudi* was seen to resemble that of *P. vinckei*, provided that contaminating organisms (e.g., *Eperythrozoon*) were removed from the system (Ott, 1968).

Landau and Killisk-Kendrick (1966) suggested the possibility that *P. chabaudi* might prove to be a subspecies of *P. vinckei*, but favored retaining it as a separate species until more information regarding the sporogony and primary exoerythrocytic schizogony of *P. vinckei* became available. To date, *P. chabaudi* is still referred to as a separate species and its taxonomic position remains uncertain.

Morphology and Ultrastructure

In the white mouse, *P. chabaudi* first appears as a small compact ring which grows in size until it occupies much of the erythrocyte. The cytoplasm of the parasite is amoeboid with the production of fine pseudopodial processes, while the nucleus assumes various shapes: spherical, bar-like or cup-shaped. It has been observed that two or more trophozoites of *P. chabaudi* may be joined together in a single host cell and this association has been termed an "accole" (Killby *et al.*, 1969). This phenomenon has been rarely seen in other rodent malarias and certainly has not been observed in *P. vinckei*. Its significance is unknown.

Electron microscopic examination of *P. chabaudi* in mouse erythrocytes revealed characteristics similar to those exhibited by other mammalian malarial parasites (Killby *et al.*, 1969).

As in *P. falciparum* (Trager *et al.*, 1966) and *Plasmodium coatneyi* (Rudzinska *et al.*, 1968), alterations in the host cytoplasm resembling Maurer's clefts were observed. The significance of these clefts is unclear, but it is possible that they serve some excretory or absorptive function. The bulges and fingerlike extensions of the parasite cytoplasm, the appearance of the food vacuoles, the sausage-shaped vacuole often broken into two or more pieces, and the variety of shapes of the nucleus, all characterize *P. chabaudi* as polymorphic. Like other malarial parasites the trophozoites and merozoites have a double unit membrane limiting their cytoplasm. The merozoites are enveloped by a triple layered membrane and possess no microtubules beneath the inner layer. This limiting membrane system at the surface of the merozoites might serve as a protective coat during their release into plasma.

Similar to *P. berghei* and *P. vinckei*, *P. chabaudi* appears to engulf host cell cytoplasm through a cytostomal structure. Killby *et al.* (1969) observed host mitochondria within the parasite cytoplasm. This is a very common finding in *P. chabaudi*, and it has been suggested that these mitochondria are broken down, with the subsequent utilization of the released host mitochondrial enzymes (Killby *et al.*, 1969). Typical protozoan mitochondria seem to be lacking in *P. chabaudi*. Digestion appears to occur in single membrane bound vesicles which contain one to several pigment granules.

## *Plasmodium gallinaceum* (Brumpt, 1935)

### Origin and Taxonomic Status

Broussais was probably the first to identify *P. gallinaceum* in domestic chickens in 1910, at Nhatrang in Indo-China, but it was Brumpt (1935) who described the organism under the name of *Plasmodium*

*gallinaceum*. Niles *et al.* (1965) found the natural vector to be *Mansonia crassipes*, but a number of mosquitoes are able to transmit the parasite experimentally. *P. gallinaceum* is capable of developing in at least six genera, *Aedes*, *Culex*, *Armigens*, *Culiseta*, *Mansonia* and *Anopheles*, but *Aedes aegypti* is commonly used in laboratory experiments. The susceptibility of *A. aegypti* is not absolute for Ward (1963) was able to breed a strain of the mosquito which was 98% resistant to infection.

The natural host of *P. gallinaceum* is the jungle fowl, *Gallus sonnerattii* being heavily favored. The secondary host is *Gallus gallus*, the domestic chicken. Until recently only a single strain (Brumpt's) of *P. gallinaceum* had been isolated. Dhanapala (1959) isolated another strain from Colombo, but this strain was identical to the original one in morphology and behavior. Under a variety of unnatural conditions, *P. gallinaceum* may undergo mutations, producing substrains, but after a number of normal transmissions most of the substrains regain the character of the type.

Morphogenesis

Huff and Coulston (1944) used *P. gallinaceum* to illustrate the existence of the pre-erythrocytic schizogony cycle which is now considered to be part of the natural developmental events in all sporozoite induced plasmodial infections. Two generations of tissue-inhabiting parasites are required before the parasite becomes capable of infecting red blood cells.

Schizogony in the blood occurs fairly synchronously as was demonstrated by Giovannola (1938). There is a steady rise in parasitemia until there may be more parasites than blood corpuscles. The intensity

of infection, however, varies according to the age of the bird. As in other malarial infections, the parasites do not disappear completely after the crisis, an occasional trophozoite being demonstrable even years later.

Ultrastructure

Like other malarial organisms, the trophozoites of *P. gallinaceum* are covered with a thin cytoplasmic membrane, which appears as a double membrane. The eccentrically located nucleus is partially electron dense containing agglomerates of small granules. In the cytoplasm of the parasite endoplasmic reticulum, food vacuoles and hemozoin pigment are visible. Both the vacuoles and pigmented areas are surrounded by a double membrane.

In mature schizonts, all the cytoplasmic organelles and the pigmented areas are apparently concentrated in a small area adjacent to the merozoites being formed. No indications of food vacuoles were noted by Ristic and Kreier (1964). All merozoites were provided with a double outer membrane, an electron dense ground substance and an irregularly shaped, usually centrally located nucleus. The exact mechanism by which the host cell plasma is engulfed is poorly understood, but pinocytosis has been incriminated (Ristic and Kreier, 1964).

## *Babesia rodhaini*

Origin and Taxonomic Status

*B. rodhaini*, a protozoan parasite of rodent erythrocytes, was isolated from the blood of the Congo tree rat (*Thallomys surdaster surdaster*) and adapted to white mice by van den Berghe *et al.* (1950). The arthropod vector was not described. However, it has been assumed

to be a hard tick. Though infections with *B. rodhaini* in white mice, splenectomized cotton rats and Syrian hamsters were light, repeated passages produced a strain in which mortality rates in infected animals were 80-90% within 8-11 days (Colas-Belcour and Vervent, 1953; Beveridge, 1953; Goodwin and Richards, 1960). The rat adapted strain frequently produces severe hemoglobinuria and anemia (Schroeder, 1966).

## Morphogenesis

With Wright's or Giemsa stains *B. rodhaini* appears as a single blue ring with a pink laterally situated nucleus. Small pear-shaped and amoeboid forms are occasionally observed. The organism appears to multiply by budding or binary fission (van den Berghe *et al.*, 1950; Beveridge, 1953).

## Ultrastructure

Electron microscopic studies of ultrathin sections of *B. rodhaini* have been carried out by Felwett and Fulton (1959) and Rudsinska and Trager (1960, 1962). Mouse erythrocytes infected with *B. rodhaini* contain parasites surrounded by a thin limiting membrane (100 Å thick) closely adjacent to the cytoplasm of the host cell. The endoplasmic reticulum is composed of small vesicles, which are surrounded by a thin membrane (100 Å thick).

The process which brings about the formation of the food vacuole involves invagination of the protoplasm to form a vacuole which contains host hemoglobin. The digestion of hemoglobin within the vacuole appears to be complete since no pigment is formed. This is a point of difference from *Plasmodium* species in which hemozoin pigment is present. Penetration of the host cell by *B. rodhaini* occurs without leakage of hemoglobin.

Rickard (1970) observed that *B. rodhaini* infected rat erythrocytes exhibited rates of oxygen uptake and glycolysis far higher than normal. However, the ratio of oxygen uptake to glucose utilization was no greater than that reported in normal erythrocytes infected with *Plasmodium* species, which suggested a greater dependence of *B. rodhaini* on anaerobic metabolism. The major pathway of glucose metabolism in *B. rodhaini* was therefore considered to be via anaerobic glycolysis to lactate (Rickard, 1970).

## Malarial Antigens and Antibodies

Studies of malarial antigens have been complicated by the intracellular habitat of the parasite and lack of an *in vitro* system for the propagation of plasmodial parasites. Malarial antigens have been detected:

(a) Free in plasma of malarious animals (Eaton, 1939; Torrey and Kahn, 1948; Cox, 1966; Smith *et al.*, 1969; Lykins *et al.*, 1971).

(b) Inside and on the surface of parasitized erythrocytes at certain developmental stages of the parasite (Zuckerman and Ristic, 1968).

(c) In association with various organ tissues (Ward and Conrad, 1966; Allison *et al.*, 1969; Ehrich and Voller, 1972).

Eaton (1939) reported a soluble malarial antigen in sera of monkeys infected with *Plasmodium knowlesi*, which fixes complement with immune serum. Injection of this serum antigen into normal monkeys stimulated the production of specific complement fixing antibodies which reacted with antigens extracted from parasitized cells as well as with the antigen present in serum obtained during the acute phase. A position of the antigen, termed soluble parasite substance, was precipitated

with the globulins when serum was fractionated by ammonium sulphate (Eaton, 1939). No antigen was detected in the urine of infected animals.

Torrey and Kahn (1949) extracted precipitinogen from the plasma of ducks acutely infected with *P. lophurae* by precipitation with trichloracetic acid. Contrary to the observations of Eaton (1939), these workers observed that antigen precipitated at pH 3.2 remained serologically stable for a period of 4 months. Eaton (1939) reported that antigenic activity was lost when the pH was lowered below 5.5.

Todorovic *et al.* (1968) found malarial antigens in the sera of chickens acutely infected with *P. gallinaceum*. The antigenic component appeared to be proteinaceous in nature. Serologic reactions were shown to occur between the antigen and convalescent homologous sera, as well as sera from humans, monkeys and rats which had recovered from plasmodial infections.

Wilson and Voller (1970) reported the presence of heat stable antigens which they termed malarial S-antigens (antigens which retain activity after treatment at 100 C for 5 minutes) in sera of South American owl monkeys, infected with Malayan camp strain of *P. falciparum*. Malarial S-antigens, identical with those antigens found in the sera of West African children, were found at the height of infection (Turner, 1967). Precipitating antibodies to the S-antigens were reported to appear 2-3 weeks later.

Smith *et al.* (1969), working primarily with serum soluble antigen obtained from *P. gallinaceum* infected chickens and antisera obtained from recovered chickens, used a double-diffusion in gel-precipitation assay to demonstrate the soluble antigen-antibody reactions. The antibody specific to this antigen was found in the IgG immunoglobulin class. Turner and McGregor (1969), working with *Plasmodium falciparum*,

also found that antibody activity was generally associated with the IgG class, but some gel precipitin antibodies of lgM type were identified. Findings in regard to antibody activity in the lgA class were inconclusive.

Lykins *et al.* (1971), in an extension of the work of Smith *et al.* (1969), found three precipitating serum soluble antigens in the sera of infected chickens. The relative molecular weights of the three antigens based on elution from Sephadex G.200, were S.A. 1:$5x10^5$-$1x10^6$, S.A. 2: $5x10^5$-$2.5x10^5$ and S.A. 3:<70,000. SA2 was selectively precipitated by dextran sulfate indicating that it contained a lipid component.

Turner (1967) obtained antigens of *P. falciparum* by taking placental blood of Gambian women at time of parturition and disintegrating the blood cells by use of the Hughes press. Fractionation of solubilized erythrocytes was performed by Sephadex G-200 column chromatography. Two types of antigens, designated $\alpha$ and $\beta$, were found. The $\alpha$ antigen was associated with the macroglobulin peak and its estimated molecular weight was between 300,000-900,000. The $\beta$ antigen was eluted in the same peak as hemoglobin.

More recently, Stutz and Ferris (1971) used whole blood in the Ouchterlony test for precipitins in chickens acutely infected with *P. gallinaceum*. This blood was found to be just as satisfactory as extracted serum soluble antigens, suggesting the presence of antigens on the surface of the red blood cells.

## Specificity of Malarial Antigens

Cross reactions between different species of malarial parasites and other hemosporidian infections, for example *Babesia spp.*, have been

reported by several workers. Cox (1966) detected antigens in the sera of monkeys with acute *P. knowlesi* infection which reacted in serologic tests with sera of human volunteers recovered from *P. falciparum*, rats recovered from *P. berghei*, hens recovered from *P. gallinaceum* and monkeys recovered from *P. knowlesi*. When injected into rats, the antigen induced immunity to *P. berghei* infection.

Kielmann *et al.* (1970) employed *P. gallinaceum* parasite antigen to detect malarial antibodies in human sera, by means of the fluorescent antibody test. No differences were noted by the authors in antibody titers measured with the use of antigens from different *P. gallinaceum* strains and with *P. falciparum* and *P. cynomolgi bastianelli* parasites.

Antigens in the sera of horses with acute infections of a mixture of *Babesia caballi* and *Babesia equi* were demonstrated in gel precipitation tests with the sera of clinically recovered horses (Sibinovic *et al.*, 1965). Similar antigens in the sera of dogs with acute *Babesia canis* and rats with *B. rodhaini* were observed (Sibinovic *et al.*, 1967a).

Cox *et al.* (1968) showed serologic cross-reactions between antigens in the serum of rats infected with *P. berghei*, rats infected with *B. rodhaini*, and ducks infected with *P. lophurae* and the recovered sera from each of these three infections. Mice recovered from *P. chabaudi* were shown to resist challenge with *B. rodhaini*. Rats recovered from *P. berghei* were also resistant to *B. rodhaini* challenge (Cox and Milar, 1968).

It has been suggested (Cox *et al.*, 1968; Cox and Milar, 1968; Sibinovic *et al.*, 1967b) that antigens in the serum were probably the significant immunizing substances associated with acute babesiosis and malaria. Cohen *et al.* (1969), however, hypothesized that serum soluble

antigens do not stimulate production of protective antibody but that they may be important in inducing glomerular disease through the deposition of soluble immune complexes. Therefore the role of soluble antigens and their respective antibodies in the pathogenesis of malarial infection is yet to be defined.

## Renal Disease

Atkinson (1884), Thayer (1899), Marchiafava and Bignamm (1900) described the glomerulonephritis of chronic quartan malaria. James (1910) described acute nephritis associated with edema during *Plasmodium malariae* infections. Giglioli (1932) described advanced glomerular changes in *P. malariae* infections but only in chronic or recurrent cases. He made histologic studies of five fatal cases of malarial nephritis and suggested that the renal lesions found in acute *P. falciparum* and occasionally in *P. malariae* infection, may progress to fatality, develop into chronic kidney disease, or resolve satisfactorily.

Macgraith and Findlay (1944) studied pathologic changes in the kidneys in malaria and found that the lumina of the tubules were frequently filled with material usually described as "casts," varying in appearance from desquamated epithelium and red cells to reddish granules and spherules, the composition of which was uncertain. Spitz (1946) found hypercellularity and swelling of the glomerular tufts in 9 of a series of 50 cases of malignant tertian malaria. He also found changes in both proximal and distal tubules of the kidney.

Kibukamusoke and Hutt (1967) studied a series of 77 cases of the nephrotic syndrome occurring in Uganda and found that 31 cases were infected with *P. malariae* and all but one of these showed diffuse

proliferative or focal glomerulonephritis. Pure membranous glomerulonephritis was seen in nine cases and none of these had parasitemia.

Richard Bright (1827) suggested that inflammation of the kidney in nephritis was a morbid action directly involving the organ or a consequence of a "morbid action" elsewhere, resulting in a deposition in the kidney. From experimentation in both animals and man, this "morbid action" is now considered to be an immunological one resulting from a direct assault of antikidney (glomerular basement membrane) antibodies, or the passive deposition of circulating antigen-antibody complexes in the glomerular capillaries (Unanue and Dixon, 1968; Carpenter, 1970).

Considerable insight has been gained into the probable causes of inflammation and tissue damage which occur once antibody or antigen-antibody complexes become concentrated in the wall of the glomerular capillary filter. Such damage may be the result of activation of pharmacologically active mediators or the complement system resulting in the influx of polymorphonuclear leukocytes (Carpenter, 1970). Other factors such as the metabolic state of the glomerulus, its phagocytic mesangial cells and the degree to which the clotting system is activated, may play an important role in determining the severity and intensity of the lesion. Immunogenicity of the antigen both in quantitative and qualitative terms is critically important in production of the proper size and solubility of immune complexes (Carpenter, 1970).

The observation of Grup (1968) that circulating complexes of an IgG cryoglobulin with $B_1$ C globulin may be deposited in glomeruli of some children with acute glomerulonephritis raises the point that an exogenous antigen may not necessarily be present in all glomerular deposits.

# MATERIALS AND METHODS

## Parasites

1. *Plasmodium chabaudi*: Infected mice were obtained from Dr. Elvio H. Sadun, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C. *Eperythrozoon coccoides* contamination was detected and remedied by treating the mice used in passage with oxophenarsine hydrochloride as described by Thompson and Bayles (1966). The organism has been maintained by blood passage in mice, at weekly intervals, following methods described for *P. berghei* (Cox, 1957).

2. *Babesia rodhaini* was obtained from Dr. Paul E. Thompson, Parke, Davis & Company, Ann Arbor, Michigan. The strain was maintained by blood passage at twice weekly intervals in both white Swiss mice and Wistar rats, in the same manner as for *P. chabaudi*.

3. *Plasmodium gallinaceum* was obtained from Dr. Julius P. Kreier, Faculty of Microbial and Cellular Biology, Ohio State University, Columbus, Ohio. It was maintained by blood passage at four-day intervals, by inoculating $1 \times 10^8$ parasitized erythrocytes intravenously in white leghorn roosters.

## Experimental Animals

1. Male weanling rats, certified as *Hemobartonella muris*-free, and mice, certified as *E. coccoides*-free, were obtained from Spartan Research Animals, Incorporated, Haslett, Michigan.

2. One-day male white leghorn chicks, obtained from Rainbow Trail Hatchery, St. Louis, Michigan, were raised in departmental animal rooms until 6-8 weeks of age, before they were used for experiments.

Adaptation of Mouse *P. chabaudi* to Wistar Rats

Young rats 6-8 weeks old, weighing 100-150 g., were splenectomized under sodium pentobarbital anesthesia, following standard surgical procedures. A group of four of the splenectomized rats was held for observation for a period of three weeks to detect recrudescence of latent *H. muris* infection. Rats to be infected with the mouse strain of *P. chabaudi* were inoculated intraperitoneally with 0.5 ml. of heparinized blood of a mouse with 50% of its erythrocytes parasitized with *P. chabaudi*. When 2% or more of the red cells of these rats were parasitized, their blood was passed to other splenectomized rats using an inoculum of $1 \times 10^8$ parasitized erythrocytes (P.E.) per rat. This procedure was followed until the fourth passage when the "pre-patent" period had been reduced to two days and the rats developed a parasitemia of 10% or more. Thereafter the passages were continued in rats with intact spleens.

Blood from anesthetized infected rats was obtained by cardiac puncture. Anesthetized mice were exsanguinated by severing the brachial artery and collecting blood from the subcutaneous pocket. Ten volumes of blood were mixed with 1 volume of heparinized 0.85% NaCl solution (100 units of heparin per ml.). Plasma from blood of rats and mice was collected for serologic studies after the cells had been sedimented by centrifugation at 800 g for 10 minutes and was stored at -20 C until thawed for tests. Blood for films to be stained and for erythrocyte counts was obtained by snipping the tail of rats

or mice. Dilutions for red cell counts were made with Hayem's solution in a Sahli hemocytometer and the cells were counted microscopically in a standard Neubauer chamber. Films for parasite counts were stained with Wright's stain. For reticulocyte counts, a drop of blood was mixed and allowed to stand for 20 min. with an equal volume of 0.5% new methylene blue in 1.5% aqueous potassium oxalate before preparing the film to be counter-stained with Wright's or Giemsa stain.

Preparations of parasitized cells to be used as inocula were standardized to contain $1 \times 10^8$ parasitized cells/ml. using methods described by Cox (1957, 1958) and were given to rats or mice by the intraperitoneal (IP) route.

In order to minimize further change in the rat adapted *P. chabaudi* parasites, a number of vials of blood from 13th passage rats were stored in liquid nitrogen. When the passage strain used for the remainder of the experiments had been passed more than 20 times, it was discarded and a new passage line was initiated with 13th passage blood. Thus none of the experiments here reported were initiated with parasites that had been passed more than 20 times in the rat host.

## Test of Rat Adapted *P. chabaudi* in Mice

Inocula of both strains of *P. chabaudi* and of *B. rodhaini* were standardized at $1 \times 10^5$ P.E. for mouse experiments. Latent *P. chabaudi* infections of mice were induced by Atabrine treatment as described by Cox (1957, 1958).

## Serologic Tests

### Bentonite Flocculation Test for Serum Antigen (SA) and Antibody to Serum Antigen (ABSA)

Bentonite particles, because of their ability to absorb proteinaceous materials, were used as carriers for globulins bearing SA to detect ABSA. For detecting SA, bentonite was treated with globulins of recovered animals. Bentonite stock solution was prepared according to methods described by Bozicevich *et al.* (1960) as modified by Thoongsuwan (1971). Sensitization of the bentonite particles was accomplished by adding 10 ml. of stock bentonite suspension to 0.1 ml. of globulin appropriately diluted with 0.1 M phosphate buffered saline (PBS), pH 7.2. The mixture was stored overnight at 4 C before 1.0 ml. of 0.1% aqueous methylene blue was added. After shaking on an electric vortex shaker, the mixture was centrifuged at 800 g for five minutes, the supernatant discarded and the pellet washed twice in cold PBS, before it was reconstituted in 10 ml. of distilled water. Two tenths milliliter of 0.5% aqueous bovine serum albumin were added to stabilize the reagent.

Standardization of antigen and antibody. Globulins from plasma of chickens with acute *P. gallinaceum* infection, or from recovered birds, were precipitated with 50% ammonium sulphate and dialyzed against 0.01 M PBS pH 7.2 at 4 C until free of sulphate ions, as determined by adding 1 drop of 1% aqueous barium chloride to 0.5 ml. of globulins and redissolving the precipitate in 0.1 N HCl.

In order to determine the optimum concentration of either SA or ABSA for sensitizing bentonite, a block titration of reagent antigen and antibody was done by using dilutions of 1:10 to 1:2000. Each was

titrated against plasma from chickens with acute *P. gallinaceum* which had been pretested for serum antigen (SA) and antibody to serum antigen (ABSA), prepared as serial two fold dilutions from 1:2 to 1:8182. Dilutions of 1:1000 and 1:2000 for SA and ABSA reagent, respectively, were found to be satisfactory. Reagent SA and ABSA from rats with acute, or recovered, *B. rodhaini* infections were prepared and standardized similarly (Thoongsuwan, 1971).

Test procedure. Serial two fold dilutions of the sera or plasma to be tested were prepared in PBS, beginning at 1:2 and rising to 1:4096. The final volume of each dilution was 0.2 ml. To this was added 0.1 ml. of sensitized bentonite suspension. The mixture was agitated on a rotary shaker at 120 oscillations per minute for 40 minutes at 22 C and the test was read immediately and graded as follows:

4+ : Button-like aggregate of particles which remain intact after vigorous shaking

3+ : Aggregates disrupted in the form of bigger flakes

2+ : Aggregates disrupted in the form of small sized flakes

1+ : Aggregates on shaking disrupted in form of fine granules

- (negative) : Aggregates become completely agranular.

For positive controls known positive plasma from chickens infected with *P. gallinaceum* or rats with *B. rodhaini* were used. Tests of normal rat or chicken plasma were included as negative controls.

Hemagglutination Test with Trypsin-Treated Rat Erythrocytes

Plasma samples from rats infected with *P. chabaudi* were tested for cold active agglutinins for trypsinized erythrocytes using the technique described by Morton and Pickles (1947) as modified by Cox *et al.* (1966).

A 0.25% solution of trypsin was prepared in saline (0.85% NaCl) and the solution clarified by filtering through a Whatman No. 2 filter paper. Normal rat blood, obtained by cardiac puncture, was mixed with heparinized saline and the plasma removed after centrifugation at 800 g for 10 minutes. After washing the cells three times with saline, 0.5 ml. of cells was added to 4.5 ml. of trypsin solution and incubated at 37 C for 20 minutes. The trypsinized cells were washed three times and a 2% suspension was made in saline.

Serial dilutions of test and control plasma were made with saline and to each of these an equal volume of trypsinized cell suspension was added. Incubation was carried out at 22 C for four hours and then was continued at 4 C overnight before reading the test. The positive test was represented by an aggregate of cells which did not break up readily on mild agitation. Tests where aggregates remained intact after agitation were graded as 4+. Others were classified as 3+, 2+ and 1+ depending on the nature of aggregate. Tests in which the cells resuspended smoothly were read as negative. When interpreting the test 1+ was invariably classified as doubtful or negative since trypsinized red blood cells tend to be very sensitive to agglutination (Mann and Ristic, 1963; Cox *et al.*, 1966).

## Reagent Antisera

### Anti-*P. chabaudi* Serum

Mature rats were inoculated I.P. with $10^8$ *P. chabaudi* infected erythrocytes and 7 days after recovery from acute infection, they were given a second inoculum of $10^8$ infected cells. A third inoculum of $10^8$ cells was given 7 days later and 10 days afterwards the rats were exsanguinated. Serum was removed after keeping the blood at 4 C

overnight, and was clarified by centrifugation at 800 g for 20 minutes. The globulin was precipitated with a saturated solution of ammonium sulphate (50%) and dialyzed in the cold against 0.01 M PBS (pH 7.5) until free of $SO_4$ ions. After testing for activity against *P. chabaudi* antigen in the double diffusion in gel test, the globulins were stored at -20 C.

Antiserum to Plasma of Chickens with Acute *P. gallinaceum* Infection

Plasma from the malarious chickens was tested for SA with the TBF test employing bentonite treated with reagent ABSA. Samples with high SA titers were pooled and after clarification by centrifugation at 3000 g for 30 minutes at 0 C, the plasma was used to immunize normal rats. Each rat was given 3 injections of 1 ml. of plasma at 2-day intervals and 3 weeks later the rats were bled by cardiac puncture. Serum from these rats was absorbed with normal rat erythrocytes and normal rat serum and then tested for ABSA with globulin from rats with acute *B. rodhaini* infections by double diffusion in gel tests. The serum was stored at -20 C until needed.

Antiserum to Plasma from Rats with *B. rodhaini* Infection

Plasma from rats with acute *B. rodhaini* infection was used to immunize normal rats to produce antibody to SA of babesial origin in the manner as described for plasma of malarious chickens. Serum from these rats was tested for ABSA as described above and was stored at -20 C.

## Antisera to Normal Whole Rat Serum from Rabbits and to Normal Rat IgG from Guinea Pigs

The antisera were furnished by Drs. J. F. Williams and Wes Leid of this department.

## Purification of Antigens and Antibodies

### Gel Filtration of Antigens from Plasma

Plasma from acutely malarious rats collected at the peak of parasitemia was centrifuged at 2000 g for 30 minutes to remove fibrin and lipids. It was then dialyzed for 24 hours against 0.1 M phosphate buffer pH 7.5 + 0.2 M NaCl or against 0.1 M TRIS-HCl buffer + 0.2 M NaCl. After dialysis further clarification was achieved by centrifugation as above.

Gel filtration was performed on a column of Sephadex G-200 (2.5 x 90 cm.) using 0.1 M phosphate buffer + 0.2 M NaCl, or 0.1 M TRIS-HCl buffer + 0.2 M NaCl, for elution at a flow rate of 12 ml./hr. Four milliliters of the plasma sample with sucrose added to make 3% were applied on the column, and 3 ml. fractions were collected. The optical density of each fraction was read at 280 nm, using a Hitachi Perkin Elmer (Coleman 124) spectrophotometer. Appropriate fractions were pooled as indicated in Figure 1 and the optical density (at 280 nm) of each pool was recorded. The pools were then dialyzed overnight in 1/100th molar strength of the original buffer. The protein concentration of each pool was determined according to the method of Lowry *et al.* (1951) before and after lyophilization. When reconstituting, the samples were diluted to three times that of the original plasma volume. Protein loss did not exceed 3%, and this was probably attributable to some of the protein bearing fractions not being incorporated into pools.

Figure 1. Protein concentrations as measured by optical density at 280 nm in fractions of plasma from rat with acute *Plasmodium chabaudi* infection collected after molecular sieving through a column of Sephadex G-200, and the protein bearing fractions pooled for study.

Figure 2. Protein concentrations as measured by optical density at 280 nm in fractions of solubilized *Plasmodium chabaudi* parasites obtained from rats after molecular sieving through a column of Sephadex G-200, and the protein bearing fractions pooled for study.

FIG. 1

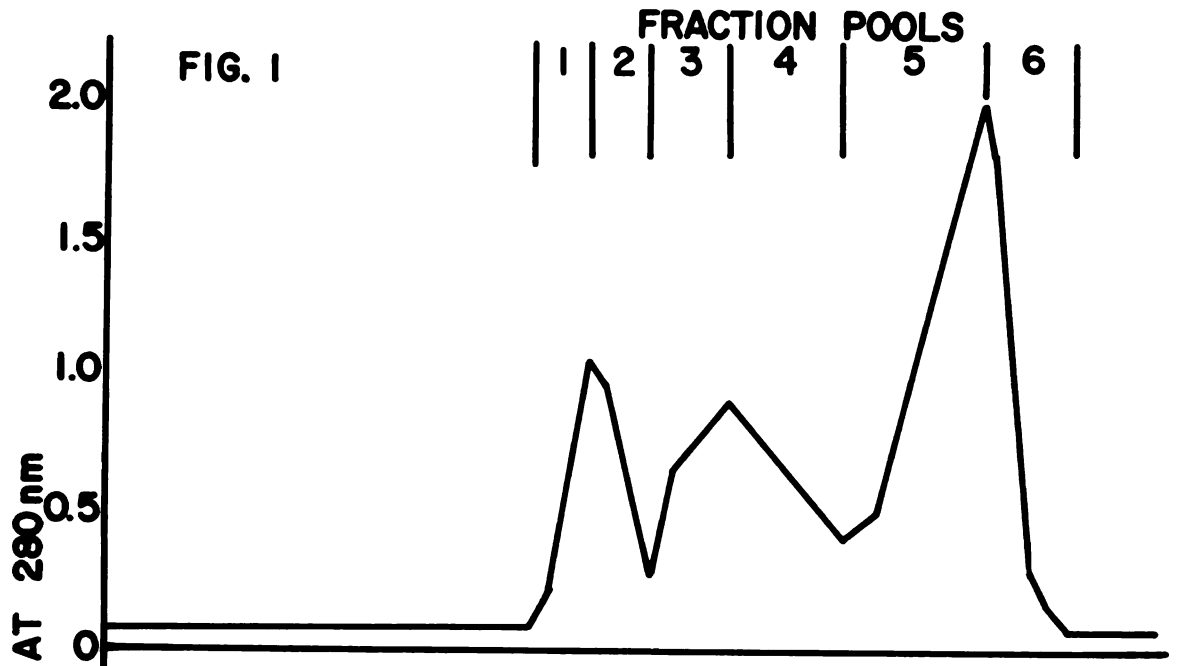
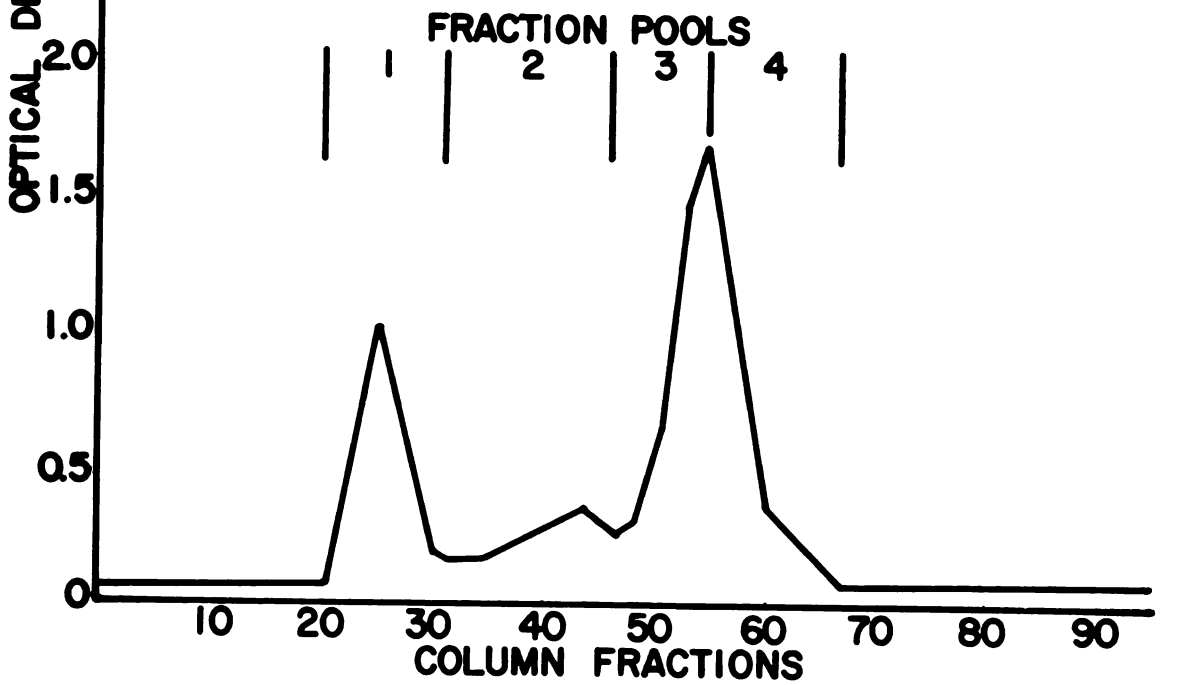

FRACTION POOLS
1
2
3
4
5
6
2.0
1.5
1.0
0.5
0
OPTICAL DENSITY AT 280nm

FIG. 2


FRACTION POOLS
1
2
3
4
2.0
1.5
1.0
0.5
0
OPTICAL DENSITY AT 280nm
10
20
30
40
50
60
70
80
90
COLUMN FRACTIONS

All pools were tested for antigen and antibody activity in double diffusion in gel tests and positive fractions were stored at -20 C.

Antigen Leached from Infected Erythrocytes

After removal of the plasma, infected erythrocytes taken from animals at the peak of parasitemia were washed three to five times, at a controlled temperature of 22 C, in 0.85% NaCl until the supernatant was free of protein. An equal volume of 0.2 M NaCl (hypertonic solution) was added and the mixture was incubated at 4 C overnight. The mixture was then centrifuged at 1000 g for 15 minutes. The supernatant was then dialyzed overnight against several changes of PBS. After concentration by ultrafiltration, the material was again dialyzed against PBS for 3-4 hours. It was then tested for antigen in double diffusion in gel test against *P. chabaudi* antiserum. Tests for antibody were made by immunoelectrophoresis using anti-rat serum and anti-rat IgG. Samples were stored at -20 C until needed.

Parasitic Antigen Preparations

The infected blood cells obtained from rats at the peak of *P. chabaudi* parasitemia were washed several times in cold normal saline and parasitic antigen was prepared according to methods described by Chavin (1966), Cook *et al.* (1969), and Aikawa *et al.* (1972). Most of the leukocytes were removed by passing washed cells through a column (2.5 cm. x 50 cm.) packed with glass beads (2 mm. in diameter). Infected cells were concentrated using a discontinuous sucrose gradient method. Gradients were prepared in 20 ml. glass tubes using sucrose concentrations of 0.3 M to 1 M sucrose in 0.85% NaCl or Ringers solution in accordance with methods described by Brakke (1953), Miller *et al.* (1971) and Cox (1970). The gradients were prepared in an ice bath

using a volume of 3.0 ml. of each solution. Three milliliters of a 20% suspension of the cells were applied to the top of the gradient by running the sample along the wall of the tube. The tubes were allowed to sit in swinging bucket rotors in the centrifuge (at 4 C) for about 5 minutes to allow for temperature equilibration, prior to centrifugation at 5000 g for 2 minutes. Parasitized cells, settling between 0.5 M-0.6 M, were removed from the top and washed twice in 0.85% NaCl before being used for parasite antigen preparation.

A volume of cells was added to 5 volumes of a 1% solution of saponin, and incubated for 30 minutes at 37 C before being centrifuged at 3000 g for 1/2 hour. The pellet was washed repeatedly with cold PBS pH 7.0 until the supernatant was free of visible hemoglobin. The washed pellet was suspended in twice its volume of 0.1 M veronol acetate saline buffer (pH 8.6) and sonicated twice for two minutes at 0 C with a five minute interval between each sonication. An appropriate amount of veronal acetate buffer (approximately 5 times the volume of the pellet) was added and the mixture was stirred at 4 C for 24 hours. The supernatant, collected after centrifugation at 3000 g for 45 minutes, was dialyzed against several changes of 0.01 M PBS pH 7.5 before lyophilization, and the lyophilized extract was reconstituted in 0.01 M PBS pH 7.5 and tested for activity against recovered serum. Samples were stored at -20 C.

Column chromatography of the parasitic extract was performed using the method described for acute plasma.

Study of Kidney Tissues for Bound Antigen and Antibody

Kidney tissues of malarious rats were studied for the presence of complexed antigen and antibody according to methods described by

Allison *et al.* (1969), Gallo (1970) and Banks *et al.* (1972). Kidneys collected at the peak of infection were washed in cold PBS and were homogenized in PBS pH 7.0. The supernatant, obtained after centrifugation at 800 g for one hour, was tested for the presence of *P. chabaudi* antigen in the double diffusion in gel test against recovered serum. The sediment was washed 6-8 times with cold PBS (pH 7.0) and centrifuged at 2000 g for 30 minutes at 0 C until the supernatant was colorless.

The washed pellet was suspended in five times its volume of 0.1 M citric acid (pH 2.5) and stirred at 4 C for 24 hours. The supernatant was adjusted to pH 7.0 with 0.1 N NaOH and concentrated by ultrafiltration. The presence of immunoglobulins was investigated by immunoelectrophoresis and double diffusion in gel tests against rabbit anti-rat serum and guinea pig anti-rat IgG.

## Antigen and Antibody Analysis

### Double Diffusion in Gel Test

The double diffusion in gel test was used to test fractions of blood from malarious rats for antigen activity against recovered serum. Test slides were prepared by methods modified from those of Crowle (1961), employing 0.1 M barbital buffer pH 8.0 and 1% Noble Ion Agar (see Appendix A). About 3 ml. of agar were spread on 2.5 x 7.5 cm. acid-alcohol-cleaned slides and allowed to harden before the wells were cut. These were of either 4 or 6 mm. diameter and 8 mm. apart from the central and other peripheral wells. The wells were then filled with the reactants and refilled 2 or 3 times within the first 6 hours. The slides were incubated at 22 C and examined for precipitin lines at 48 and 72 hours. After incubation, test slides were washed in several changes of phosphate buffer + 2% NaCl over 48 hours (Williams and Chase,

1968). The dried gels were stained with Amido black, following the method described by Crowle (1968) (see Appendix B).

Immunoelectrophoresis

Immunoelectrophoretic studies of blood components of malarious rats were performed according to Kabat and Mayer (1971) employing high resolution buffer (0.05 M TRIS-Barbital Na-barbital pH 8.8) obtained from Gelman Instruments Company of Ann Arbor, Michigan. Gel slides were prepared as for the double diffusion in gel test, and a current of 3 ma per slide was applied for 90 minutes. Troughs were filled with appropriate antisera and precipitin lines were allowed to develop for 48-72 hours at 22 C. The slides were then washed and stained as described for the double diffusion in gel test.

Disc Electrophoresis

The physicochemical nature of blood fractions and urine of malarious rats was determined by disc electrophoresis of the material following methods described by Ornstein and Davis (1964), Chavin (1966) and Sherman (1965). For the components of the running gel, sample gel, spacer gel, and buffers, see Appendix C.

Staining procedures after disc electrophoresis

Protein. Gels were stained for 15 minutes in a solution of 0.1% Amido Black[1] in equal volumes of 12% acetic acid and 1.6% sodium acetate (4.5 ml. each) plus 10 ml. glycerol. Destaining was done in 2% acetic acid.

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[1]Amido Black NBR, Allied Chemical, Morristown, N.J.

Carbohydrate. The gel was first fixed in 7.5% acetic acid for one hour and then placed in freshly prepared 0.2% periodic acid in water at 4 C for one hour. Periodic acid was removed by washing with several changes of 7.5% acetic acid, after which the gel was stained with Schiff's reagent at 4 C for three hours. Repeated washing in potassium meta-bisulphite and 1 N HCl removed the nonspecific background. The gel was stored in this solution.

Lipid. A saturated solution of Sudan Black[1] in 100 ml. of 60% ethanol at 37 C was used. Staining was carried out for 30-60 minutes and 50% ethanol was used for differentiation.

DNA Feulgen. The gel was placed in ice cold 1 N HCl for 30 minutes, hydrolyzed in 1 N HCl at 57 C for 12 minutes and then washed in cold 1 N HCl. The gel was transferred to Schiff's reagent at room temperature and left until positive. Repeated washings with the sulphite rinse were done as for carbohydrate. The gel was stored in bisulphite rinse.

Controls. Positive controls for lipid, carbohydrate, and DNA were treated and stained similarly.

## Histopathology

Groups of 4 rats were exsanguinated daily from day 1 through day 14 after infection with *P. chabaudi*. Kidney tissue samples, not more than 3 mm. in thickness, were collected and fixed in 10% phosphate buffered formalin (pH 7.0) or Bouin's fixative. The tissues fixed in Bouin's solution were removed after six hours and washed in 50% alcohol

[1]Sudan Black B, Allied Chemical, Morristown, N.J.

to remove excess picric acid, before being stored in 70% alcohol. Afterwards these and tissues fixed in formalin were processed and embedded according to Luna (1968). Dehydration was accomplished with successive treatments of 1 hour in 70% ethanol, 1 hour in 95%, 2 hours in absolute ethanol, 2 hours in xylol for cleaning and 1 hour in paraplast paraffin at 57 C. Each piece of tissue was oriented and mounted in a block of paraffin. The sections were cut 4-5 μ in thickness. The glass slides on which tissue sections were mounted were covered with a thin layer of Mayer's egg albumin. The sections were stained with either hematoxylin-eosin or Giemsa stain. Photomicrographs were taken with a Zeiss microscope using Ektachrome ASA160 film.

The severity of renal damage was evaluated according to a system designed to give a quantitative assessment (Iturri and Cox, 1969). A score of 1+ through 4+ was given to each of 100 glomeruli. The glomeruli and adjacent tubules in a "very acute" stage were designated 4+ while near normal nephrons were scored as 1+. Any changes in between were designated as 3+ or 2+. Normal glomeruli and adjacent tubules were scored 0. The sum of the scores of 100 nephrons was used to indicate the severity of kidney damage (SKD). Also the glomeruli were evaluated on the basis of a decrease or an increase in the number of nuclei in glomerular tufts according to the method described by Kibukamusoke and Hutt (1967). Counts were made of nuclei in each of 100 glomeruli and the average number per glomerulus was used to evaluate the condition of the kidney.

## Statistical Analysis

Two-way analysis of variance was used to assess the statistical significance of variation within the animals and also variations within

the days. Both the quantitative evaluation of the kidneys and the average number of nuclei in glomerular tufts were treated as a "Randomized Complete Block Design", taking the number of animals as "replicates" and number of days as "treatments."

The least significant difference (LSD) was calculated using the following expression:

$$LSD = t\sqrt{\frac{2\ (\text{Error Mean Square})}{\text{No. of Replications}}}$$

## Urine Analysis

A group of four rats each infected with $10^8$ *P. chabaudi* infected erythrocytes was maintained in metabolism cages and urine was collected every 24 hours. Proteinuria was estimated by adding an equal volume of cold 10% trichloroacetic acid (TCA) to the urine sample and dissolving the precipitated protein in 0.1 N NaOH. The protein concentration was determined following the method of Lowry *et al.* (1951). A group of four normal rats injected with normal cells ($3 \times 10^8$) was used as controls. Immunoelectrophoresis and double diffusion in gel tests were performed to detect serum proteins and *P. chabaudi* antigen.

## EXPERIMENTAL RESULTS

### Adaptation of *Plasmodium chabaudi* to Rats

Wright's-stained blood smears prepared daily from each of the young splenectomized rats used to initiate the *P. chabaudi* infection indicated that the rats had a patent parasitemia from the inoculum after one day. These parasites disappeared by the second day and no more were seen until the sixth day. The parasitemia remained patent for 4 days and attained a peak of 7% PE. Splenectomized rats of the fourth passage exhibited increases in parasitemia on the second day after inoculation and during the 4 days of patent infection a peak parasitemia of 30% PE was attained. Thereafter, passage was carried in rats with intact spleens. The first intact rat passage had a prepatent period of 6 days and the parasitemia, which peaked at 2% PE, remained patent for 2 days. Rats of the tenth passage were fully grown. Patent infection which developed 2 days after inoculation remained patent for 6 days and peaked at 28% PE (Table 1).

### Comparison of the Affinity for Normal Erythrocytes of the Rat Adapted and Mouse Strains of *Plasmodium chabaudi*

In the study of the extent to which rat and mouse strain *P. chabaudi* infected reticulocytes, it appeared that neither strain had marked affinity for immature cells, though parasites were seen in reticulocytes when parasitemia was high and there was marked reticulocytosis (Table 2).

Table 1. Averages of prepatent time in days, duration of patency and peak percentage of parasitized erythrocytes (percent PE) during initial passages of mouse strain *Plasmodium chabaudi* in splenectomized rats and subsequently in intact rats

| Passage # | | Days before patent parasitemia | No. days parasitemia patent | Peak percent PE |
|---|---|---|---|---|
| 1 | splenectomized | 6 | 4 | 7 |
| 2 | splenectomized | 4 | 3 | 13 |
| 3 | splenectomized | 2 | 4 | 11 |
| 4 | splenectomized | 2 | 4 | 30 |
| 1 | intact | 6 | 2 | 2 |
| 2 | intact | 5 | 3 | 9 |
| 3 | intact | 6 | 2 | 11 |
| 4 | intact | 6 | 3 | 10 |
| 5 | intact | 4 | 3 | 13 |
| 6 | intact | 3 | 4 | 20 |
| 7 | intact | 3 | 5 | 23 |
| 8 | intact | 2 | 6 | 17 |
| 9 | intact | 3 | 5 | 23 |
| 10 | intact | 2 | 6 | 28 |
| 11 | intact | 2 | 5 | 31 |
| 12 | intact | 2 | 5 | 28 |
| 13 | intact | 2 | 5 | 35 |

Table 2. Percentage of mature erythrocytes and reticulocytes infected during the course of rat adapted *Plasmodium chabaudi* and during the course of mouse *P. chabaudi* infection

| Day of Infection | Total percent parasitized erythrocytes | Total percent reticulocytes | Percent reticulocytes parasitized |
|---|---|---|---|
| Rat strain *P. chabaudi* | | | |
| 1 | 0 | 4.3 | - |
| 2 | 1.0 | 4.1 | - |
| 3 | 8.7 | 4.3 | <0.1 |
| 4 | 13.0 | 4.5 | <0.1 |
| 5 | 36.0 | 6.0 | <0.1 |
| 6 | 21.0 | 17.0 | 11.3 |
| Mouse strain *P. chabaudi* | | | |
| 1 | 0 | 4.5 | - |
| 2 | 6.0 | 5.0 | <0.1 |
| 3 | 26.0 | 5.3 | 3.0 |
| 4 | 40.0 | 6.9 | 7.0 |
| 5 | 67.0 | 19.0 | 45.0 |

## Immunopathologic Studies of *P. chabaudi* Infections of Rats

A group of 6 infected rats served as the source of data for the percent PE and RBC counts throughout the experiment. A second group served as a source for 4 rats to be sacrificed for blood samples and postmortem study at daily intervals after infection. Blood for smears to determine percent PE and for RBC counts was obtained before exsanguination. The spleen and kidney tissue were taken at postmortem examination. The spleen volume in milliliters was determined by water volume displacement and spleen and kidney tissues were preserved for future study. Four normal rats were sacrificed for controls.

The data on RBC counts, percent PE, spleen volume and agglutinin for trypsinized erythrocytes from this experiment are summarized in Figure 3. Parasitemia reached a mean peak value of 35% PE. Mean RBC counts were reduced from an average of $6.7 \times 10^6$ to $3.0 \times 10^6$ per cu.mm., representing a loss of 50% of the circulating erythrocytes. The presence and titer of agglutinin for trypsinized rat erythrocytes were generally associated with the onset, peak and recovery from anemia. Splenic enlargement became evident with the onset of blood loss and was maximal during the time of the parasitemia-anemia crisis, i.e., the sudden loss of red cells and parasites.

Plasma samples taken from rats brought to necropsy were found not to have serum antigen and recovered rats did not have antibody to serum antigen.

## Histopathologic Studies of Kidney Tissues

Data obtained from histologic studies of sectioned and stained kidney tissues of infected rats autopsied at daily intervals are shown in Table 3. The average SKD value determined for the 4 rats taken

Figure 3. Red blood cell counts (RBC x $10^6$) per cu.mm., the percentage of parasitized RBC, spleen volume in milliliters, and the reciprocal of the titers of hemagglutinin for trypsinized erythrocytes in blood from rats during the course of acute *Plasmodium chabaudi* infection.

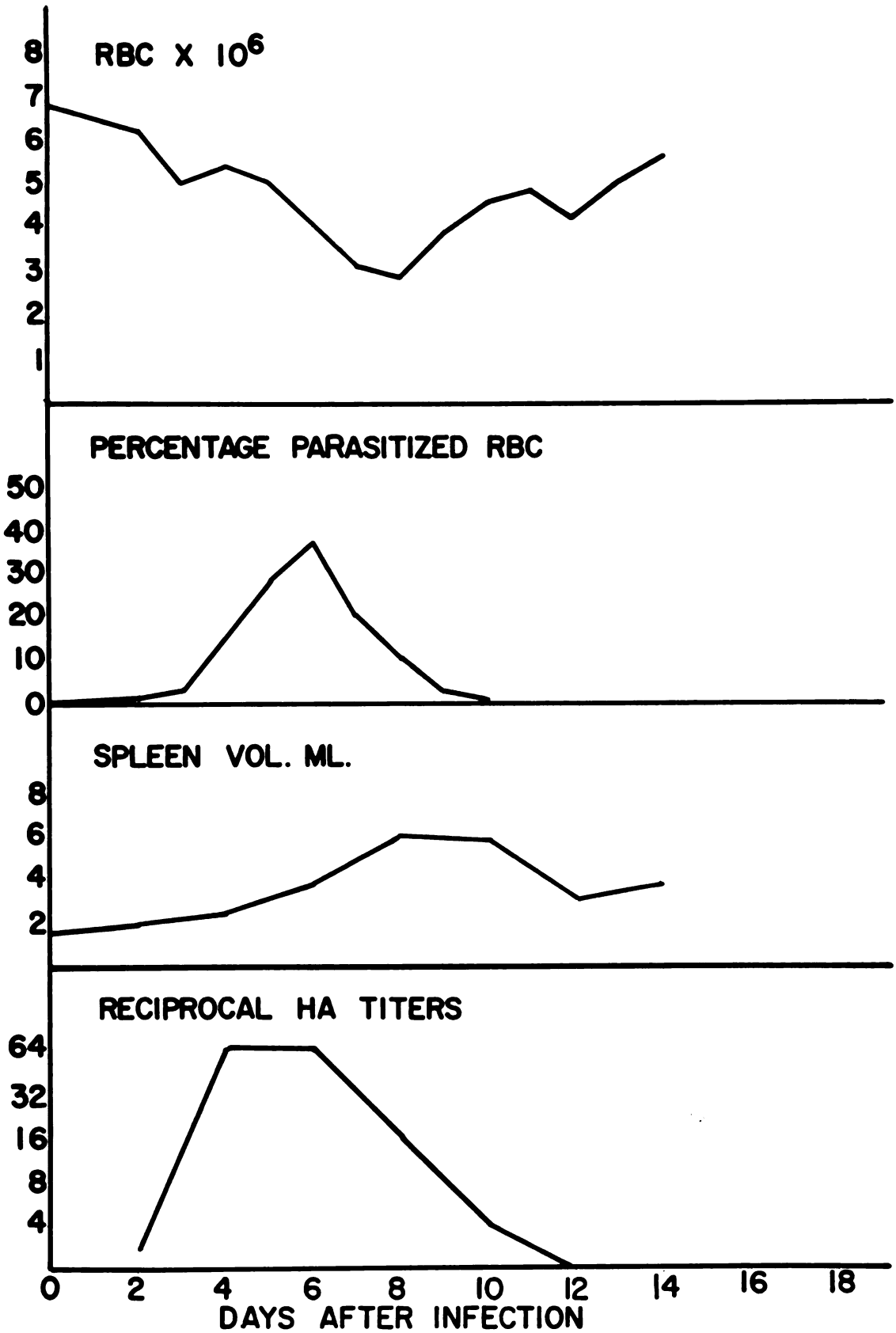

RBC X 10^6
8
7
6
5
4
3
2
1
PERCENTAGE PARASITIZED RBC
50
40
30
20
10
0
SPLEEN VOL. ML.
8
6
4
2
RECIPROCAL HA TITERS
64
32
16
8
4
0
2
4
6
8
10
12
14
16
18
DAYS AFTER INFECTION

Figure 3

Table 3. The average score of severity of kidney damage (SKD) and average number of nuclei in the glomerular tuft (NGT) found in rats brought to postmortem examination at daily intervals during the course of acute *Plasmodium chabaudi* infection

| Days | SKD[1] | No. nuclei in glomerular tuft (NGT)[2] |
|---|---|---|
| 0 | 35 | 49 |
| 1 | 38 | 53 |
| 2 | 37 | 52 |
| 3 | 36 | 63 |
| 4 | 57 | 74 |
| 5 | 74 | 76 |
| 6 | 120 | 87 |
| 7 | 109 | 76 |
| 8 | 86 | 78 |
| 9 | 74 | 69 |
| 10 | 52 | 61 |
| 11 | 49 | 63 |
| 12 | 52 | 59 |
| 13 | 43 | 61 |
| 14 | 47 | 59 |

[1]Least significant difference (LSD) for SKD = 39.

[2]Least significant difference (LSD) for NGT = 56.

randomly on each day rose from 35 on day 0 to 120 on day 6 with the greatest rate of increase after day 3. This change was also reflected by an increase in the number of nuclei counted in the glomerular tuft. After the 6th day the estimates for the SKD values gradually subsided until they reached near normal levels by days 13 and 14. There was also a gradual reduction in the number of nuclei, but the number found on days 13 and 14 were still elevated over that found on day 0. Statistical analysis indicated that SKD values greater than 39 and nuclei counts greater than 56 were significantly greater than normal (Table 4).

The changes observed in kidney tissue are shown in Figure 4. Figure 4a illustrates the appearance of a glomerulus and adjacent tubule as found in rats autopsied on day 0. The glomerular tuft is not enlarged, Bowman's capsule is of normal thickness and the adjacent convoluted tubules are patent with smooth epithelial linings. Such a glomerulus would be given an SKD value of 0, and it may be noted that there are approximately 30 nuclei visible in the tuft. Figure 4b illustrates a glomerulus evaluated with an SKD of 4. Approximately 90 nuclei were counted in the tuft. It should be noted that the lumen of the capillary is not evident and that the tuft is swollen to the extent that Bowman's space is completely occupied. The epithelial cells lining the adjacent tubules are swollen to the extent that the lumen appears to be closed. This finding was more common in rats autopsied on the 6th day of infection.

Figure 4c illustrates another finding from the convoluted tubules on days 5 and 6, the presence of hyaline or proteinaceous casts found with some frequency in kidneys of rats autopsied on these days.

Figure 4d illustrates findings that were often found with some frequency in rats autopsied on days 13 and 14. The swelling of the

Table 4. Analysis of variance (Lewis, 1966) of data on SKD and NGT obtained in histopathologic study of the kidney of rats autopsied at daily intervals during the course of acute *Plasmodium chabaudi* infection and presented in Table 3

Analysis of Variance Table (NGT)

| Source of variation | D.F. | S.S. | M.S. | F-ratio |
|---|---|---|---|---|
| Total | 55 | 6786.04 | | |
| Days (treatments) | 13 | 6255.48 | 481.19 | 38.5 |
| Animals (replicates) | 3 | 43.24 | 14.41 | 1.15 |
| Error | 39 | 487.32 | 12.5 | |

From table, F, (0.01) for 13 and 39 d.f. = 2.66

Variation between days is significant, but variation between animals is not significant.

Analysis of Variance Table (SKD)

| Source of variation | D.F. | S.S. | M.S. | F-ratio |
|---|---|---|---|---|
| Total | 55 | 2647.84 | | |
| Days (treatments) | 13 | 2478.59 | 190.66 | 44.76 |
| Animals (replicates) | 3 | 3.2 | 1.07 | 0.25 |
| Error | 39 | 166.05 | 4.26 | |

From table, F, (0.01) for 13 and 39 d.f. = 2.66

Variation within days is significant but variation within animals is not significant.

Figure 4. Changes observed in *glomeruli* and proximal convoluted tubules from the kidneys of rats autopsied during the course of acute *Plasmodium chabaudi* infection. A. Glomerulus and adjacent convoluted tubules of rat autopsied on day 0 of infection (normal). The clear area between the tuft and Bowman's membrane, the clear lumen of the tubules and a NGT count of 30 indicate completely normal conditions with an SKD value of 0. B. Glomerulus and adjacent convoluted tubules from the kidney of a rat autopsied on the 6th day of acute *P. chabaudi* infection. The glomerular tuft is swollen to the extent that Bowman's space is filled. The epithelium of the adjacent convoluted tubules is swollen and appears to have closed the lumen. The NGT count of 90 and estimated SKD value of 4 indicate acute "toxic" nephritis. C. Proximal convoluted tubules from the kidney of a rat autopsied on the 6th day of acute *P. chabaudi* infection. Swollen epithelium of the tubules has closed the lumen of most tubules. Dense masses seen in 3 of the tubules represent hyaline casts. D. Glomerulus and adjacent convoluted tubules found in a rat autopsied on the 13th day (post acute) of *P. chabaudi* infection. The glomerular tuft is of normal size and the lumina of the tubules are patent. However, lobation and fibrinous adherence of the tuft to Bowman's membrane and desquamation of tubular epithelium (SKD of 2) along with a NGT count of 60 indicate a condition of chronic nephritis. (All sections were Giemsa stained and photographed at 4.5 x.)

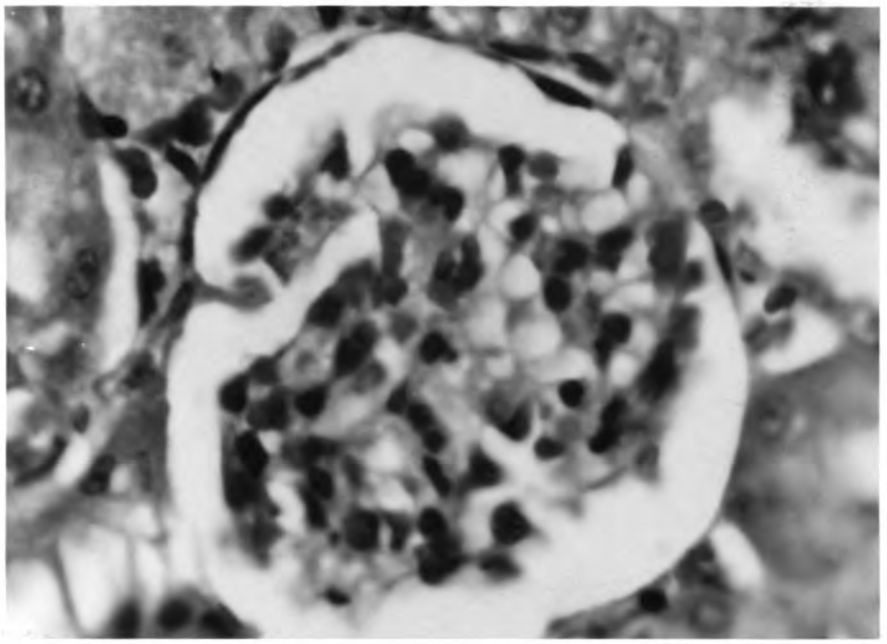


Figure 4a

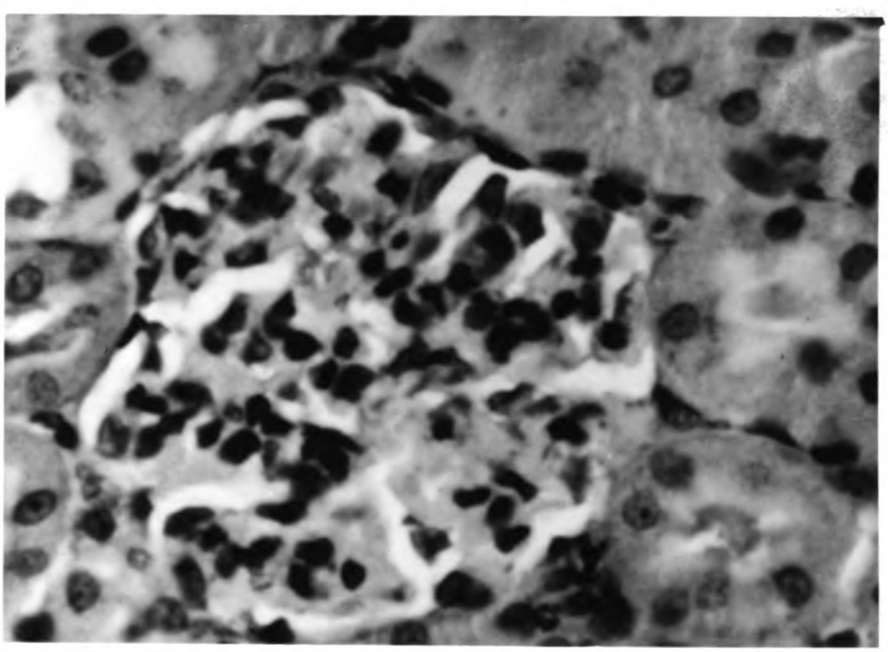


Figure 4b

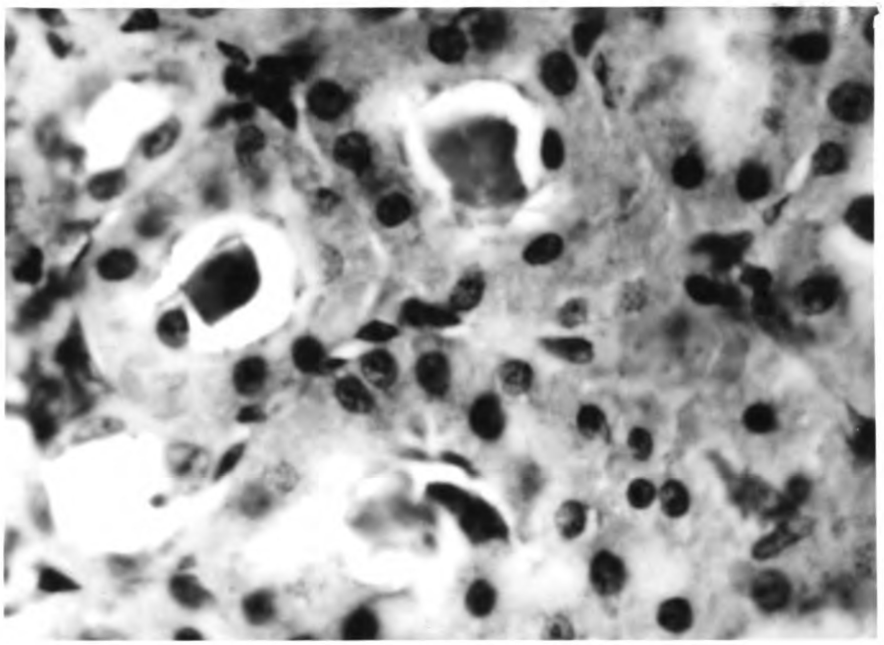


Figure 4c

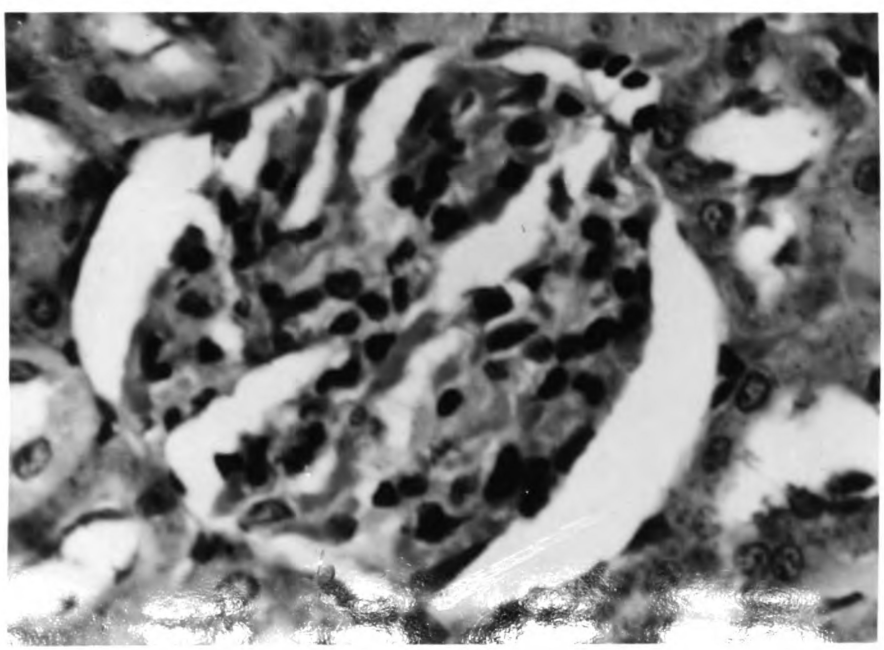


Figure 4d

glomerulus has subsided and the lumen of the capillary can be visualized. There is marked lobation of the tuft with fibrinous adherence to Bowman's membrane at several points. The adjacent tubules are patent, but desquamation of the tubular epithelium is evident. A glomerulus in this condition would be given an SKD value of 1 or 2 and the nuclei count was estimated at about 60.

Kidney Function During Acute *P. chabaudi* Infection of Rats

In order to estimate impairment to kidney function from *P. chabaudi* nephritis, 4 rats inoculated with $10^8$ *P. chabaudi* infected erythrocytes and 2 normal rats were housed in metabolic cages. Parasitemia and red blood cell counts were made and 24 hour urine samples were collected. The data from these rats are presented in Figure 5.

Parasitemia increased rapidly after the second day and peaked at 45% PE on the 5th day. Thereafter the percent PE fell sharply and infections became latent on the 10th day. A sharp fall in RBC counts began on the 3rd day and became minimal on the 8th. Thereafter the cell counts increased gradually, but had not returned to normal values on the 22nd day when RBC counts were terminated. Proteinuria increased sharply after the 3rd day to 42 mg./ml. on the 7th day. The levels then fell gradually, but became stable at 20 mg./ml. on the 13th day. Thereafter protein output in the urine remained stable for the duration of the experiment. Urine collected on the 4th day was tinged with hemoglobin and that collected on the 5th and 6th day had the color of dark port wine (Figure 6). By the 9th day the urine of the infected rats had reverted to normal color.

Protein precipitated from the urine of the infected rats was subjected to immunoelectrophoretic study using anti-rat whole serum and

Figure 5. Percentage of parasitized erythrocytes, red blood cell counts per cu.mm. (RBC x $10^6$), proteinuria in mg./ml., and hemoglobinuria in rats during the course of acute *Plasmodium chabaudi* infection. Dotted lines represent the parameters in normal animals.

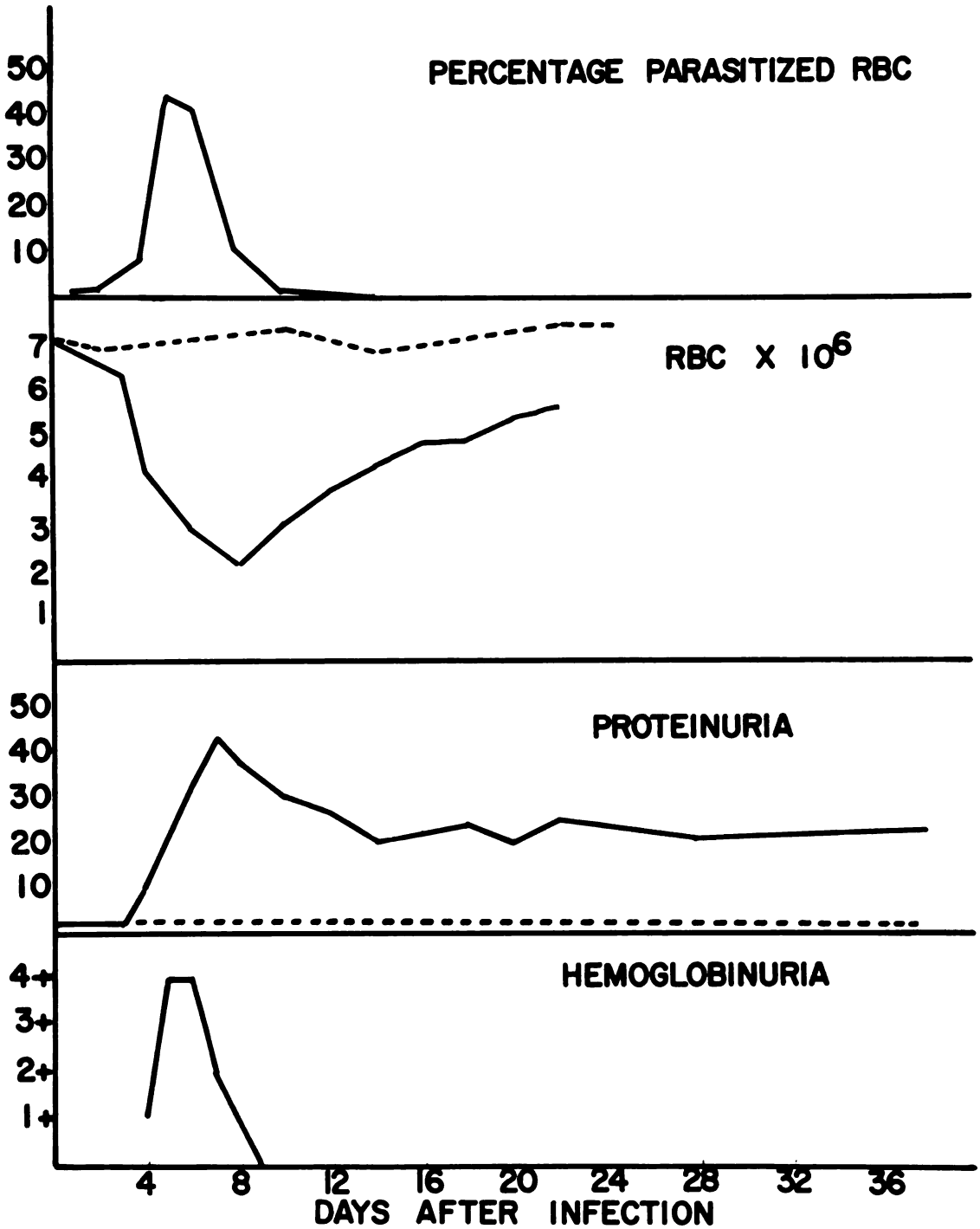

PERCENTAGE PARASITIZED RBC
50
40
30
20
10
RBC X 10^6
7
6
5
4
3
2
1
PROTEINURIA
50
40
30
20
10
HEMOGLOBINURIA
4+
3+
2+
1+
4
8
12
16
20
24
28
32
36
DAYS AFTER INFECTION

Figure 5

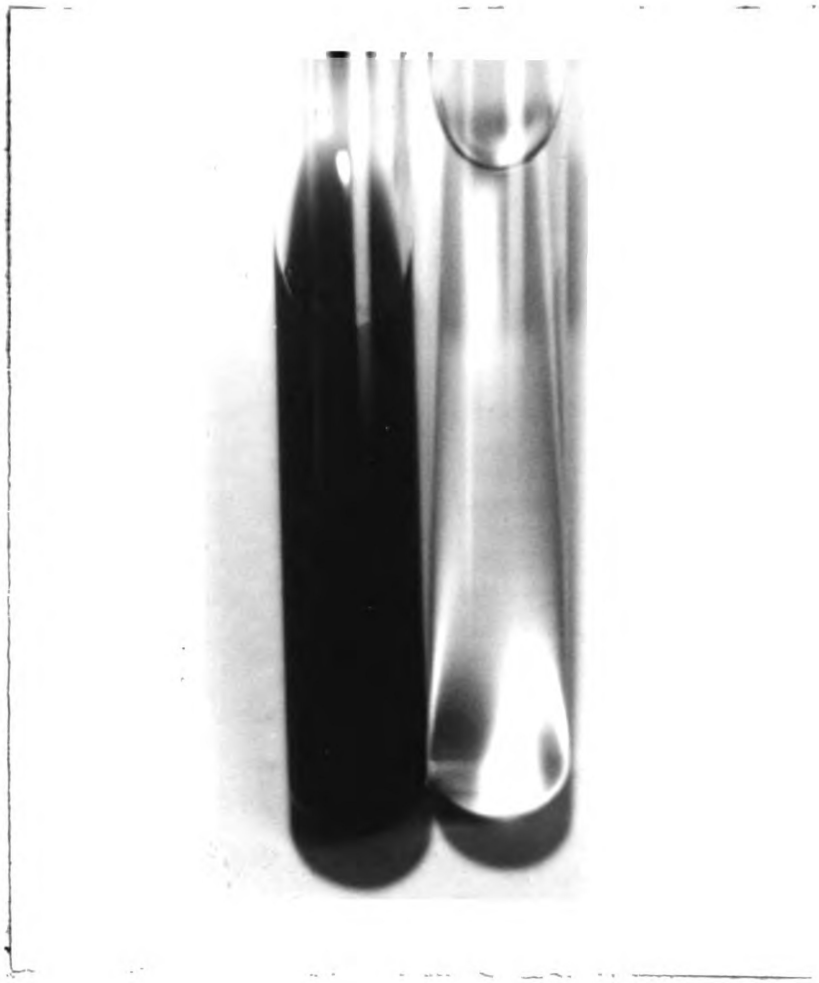


Figure 6. Photograph of urine from control rats (right) and urine collected from rats on the 6th day of acute *Plasmodium chabaudi* infection.

anti-rat gamma globulin (IgG). Comparison of the urine protein with whole rat serum using anti-rat serum reagent indicated that 5 serum components were secreted in the urine. Of these, albumin and an IgG (gamma-1 fraction) were identified (Figure 7). In disc electrophoresis 8 protein bands were detected (Figure 8). Tests for deoxyribonucleic acid, carbohydrate and lipid were all negative.

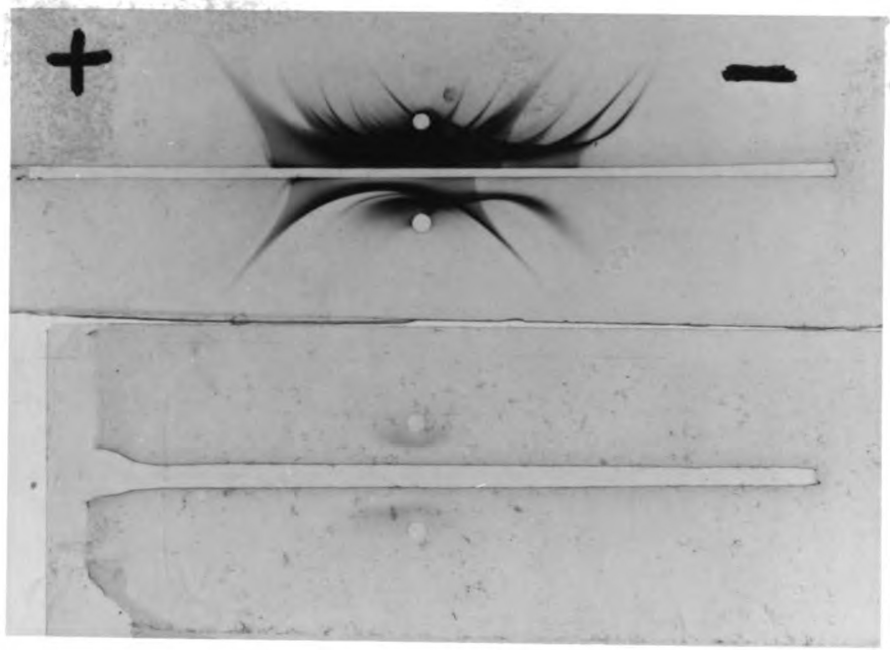
The urine protein was also tested for antigen in double diffusion in gel tests using globulin from rats hyperimmunized by *P. chabaudi* infection. The tests indicated that soluble antigen, which reacted with globulin of rats immune to *P. chabaudi*, was secreted in the urine on days 4-6 (Figure 9).

## Comparison of the Immunopathologic Responses of the Rat and Mouse Strains of *P. chabaudi* in Mice

Data on the percent PE, RBC counts, HA titers and the titers of ABSA produced by infections with the rat strain of *P. chabaudi* and the mouse strain of parasite in mice are presented (Figure 10). In this experiment the infecting inocula were reduced to $10^5$ PE. Three mice with each infection were exsanguinated on each day of the experiment. Plasma from each group of 3 mice was pooled for serologic tests.

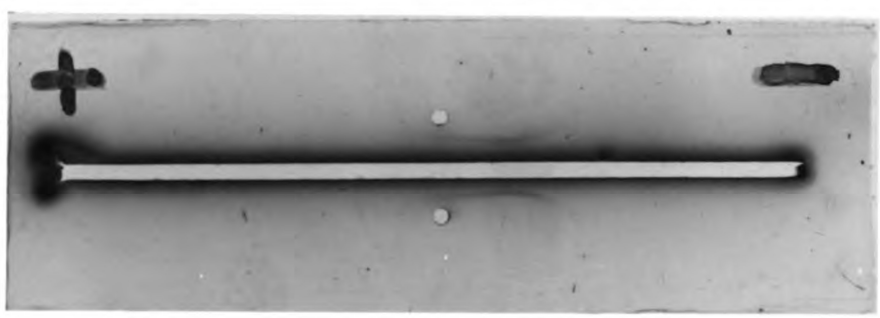
Infections with rat strain parasites developed more rapidly by two days than did infections with those of the mouse strain. This rapid parasite growth was accompanied by earlier signs of blood loss, and death of the infected animals. In both infections there was reduction in parasitemia associated with blood loss, and higher HA titers. ABSA was not detected in plasma pools collected from mice infected with the rat strain, but was present in the pool collected from mice infected with mouse parasites sacrificed on the seventh and eighth days.

Figure 7. Immunoelectrophoretic comparison of protein from urine collected on the 6th day of acute *Plasmodium chabaudi* infection and normal rat serum. A. Reaction with rabbit anti-rat serum indicates that urine contained 5 components found in normal serum. In "B" a faint precipitin line of reaction with anti-rat serum confirms the presence of the trace of protein consistently found in urine of normal male rats (Figure 4). C. Reaction with guinea pig anti-rat IgG shows a single precipitin line in the urine protein.



A

B



C

Figure 7

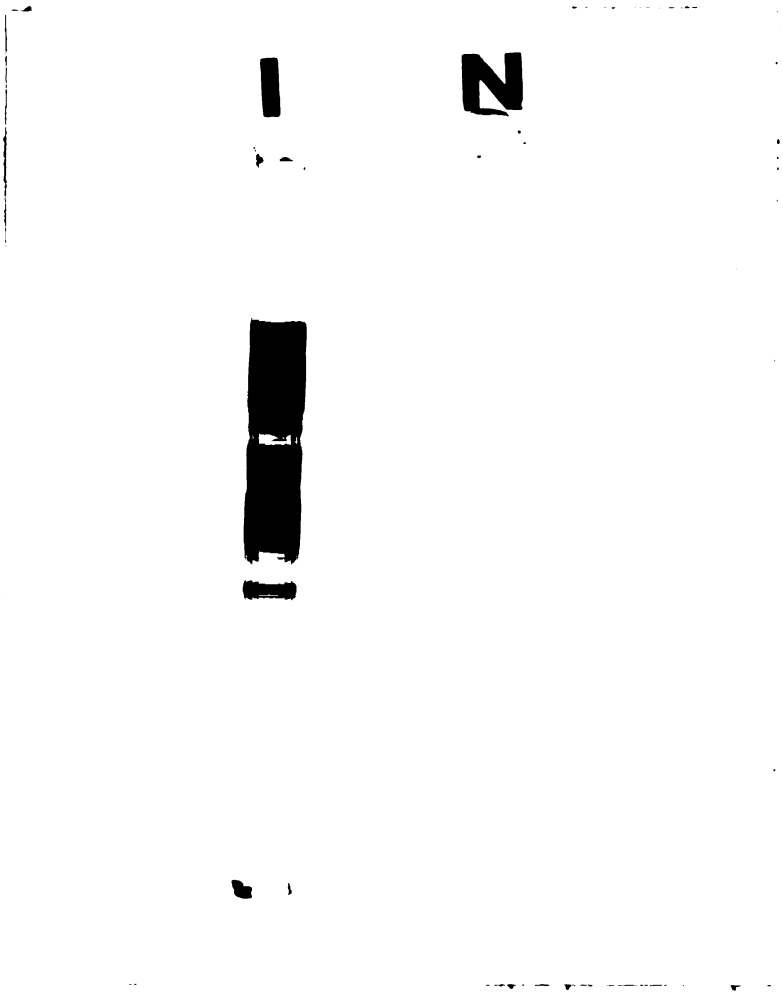


Figure 8. Gels from disc electrophoresis of protein from urine of *Plasmodium chabaudi* infected rats on the 6th day. Eight bands of proteinaceous material were detectable. One band found in urine protein of normal rats was also present in malarious rat urine.


Figure 9. Double diffusion in gel tests for antigen in urine collected from rats on the 6th day of acute *Plasmodium chabaudi* infection (well I) and normal rat urine (well N) using globulin of rats hyperimmunized by *P. chabaudi* infection (well R).

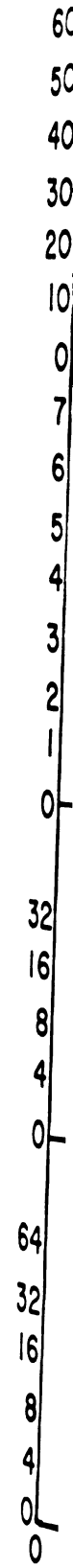



Figure 10. Percentage of parasitized erythrocytes (percent PE), red blood cell counts per cu.mm. (RBC x $10^6$) reciprocals of the titers of hemagglutinin for trypsinized erythrocytes (HA) and of antibody to serum antigen (ABSA), detected with serum antigen from the blood of chickens with acute *Plasmodium gallinaceum* infection, in the blood of mice infected with rat strain *Plasmodium chabaudi* and from mice infected with the mouse strain of the parasite.

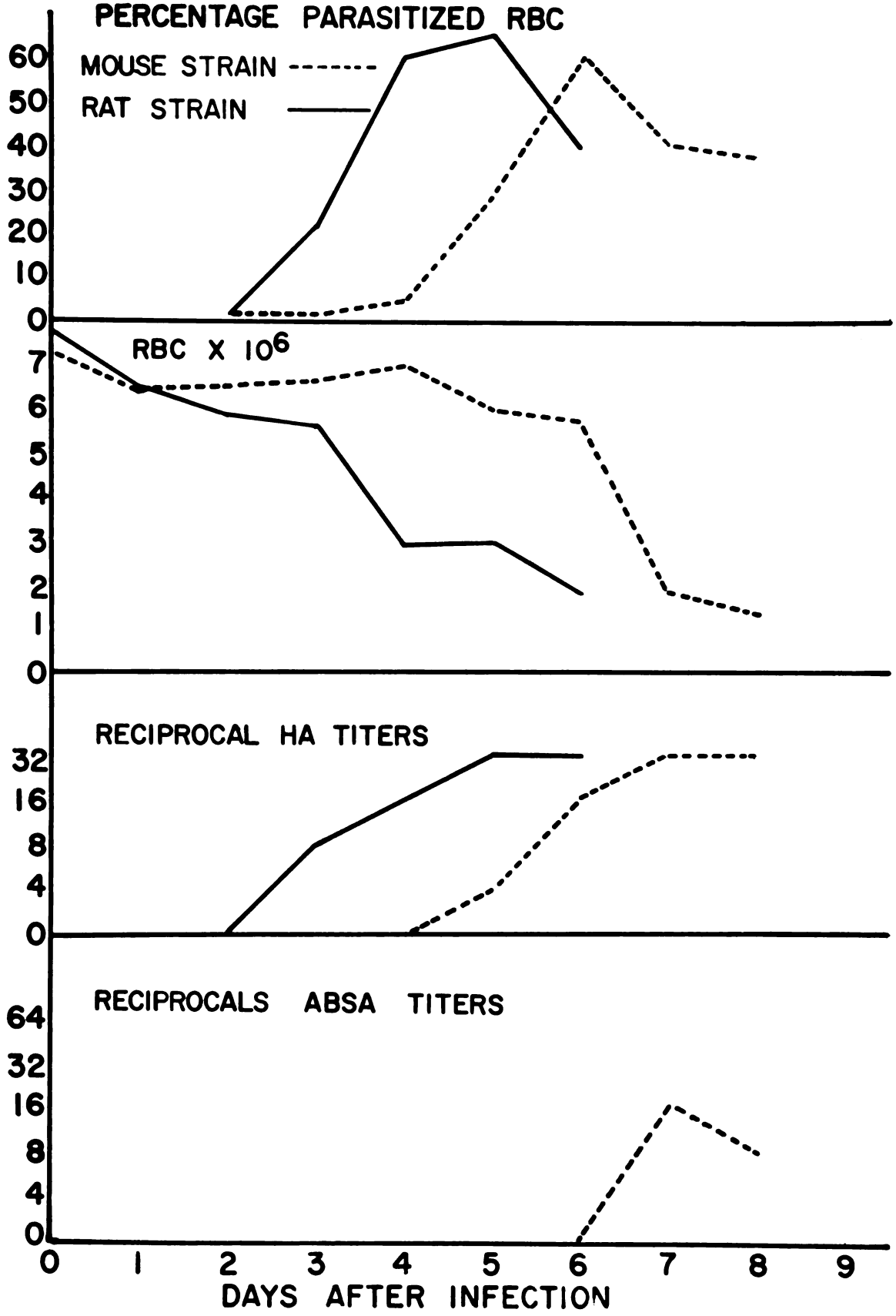

PERCENTAGE PARASITIZED RBC
MOUSE STRAIN
RAT STRAIN
60
50
40
30
20
10
0
RBC X 10⁶
7
6
5
4
3
2
1
0
RECIPROCAL HA TITERS
32
16
8
4
0
RECIPROCALS ABSA TITERS
64
32
16
8
4
0
0 1 2 3 4 5 6 7 8 9
DAYS AFTER INFECTION

Figure 10

## The Immunogenic Responses to Infection with Rat Adapted *Plasmodium chabaudi*

Eight rats recovered from *P. chabaudi* infection were divided into groups of 4. Two groups of 3 normal rats served as controls. One group of recovered and a group of control rats were infected with $10^8$ rat strain *P. chabaudi* infected rat erythrocytes. The other groups were infected with $10^8$ *B. rodhaini* infected rat cells. Data on the resulting parasitemia and mortality in these rats are shown in Table 5. The recovered rats appeared to be completely resistant to challenge with *P. chabaudi* but were no more resistant to *B. rodhaini* than were normal rats.

Mice infected with the rat strain *P. chabaudi* and given latency inducing Atabrine treatment were divided into 3 groups of 9 mice. One of the groups, along with 4 normal mice, were inoculated with $10^5$ rat strain *P. chabaudi* infected mouse erythrocytes. The second group and 4 control mice were each inoculated with $10^5$ mouse strain *P. chabaudi* infected cells while the 3rd recovered and control groups were infected with $10^5$ mouse erythrocytes infected with *B. rodhaini*. Parasitemia and mortalities were recorded. When all surviving mice had developed latent infection, they were exsanguinated and the plasma of each mouse was tested for ABSA. These data are presented in Table 6.

All mice, except one, of the group challenged with the homologous rat strain remained free of parasitemia throughout the experiment. (The parasites of the one mouse that developed parasitemia were transferred to normal mice and were kept as a passage strain.) Parasitemia in the mice challenged with mouse *P. chabaudi* was variable, ranging from a high of 1% PE in some mice to 80%. All of the mice developed patent infection

Table 5. Percentage of parasitized erythrocytes and mortality in rats challenged with rat strain *Plasmodium chabaudi* and *Babesia rodhaini* after recovery from initial rat *P. chabaudi* infection

| Rat No. | Days after challenge | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 |
| *P. chabaudi* challenge | | | | | | | | |
| 1 | - | - | - | - | - | - | - | - |
| 2 | - | - | - | - | - | - | - | - |
| 3 | - | - | - | - | - | - | - | - |
| 4 | - | - | - | - | - | - | - | - |
| *B. rodhaini* challenge | | | | | | | | |
| 1 | 7 | 50 | Dead | | | | | |
| 2 | 10 | 55 | Dead | | | | | |
| 3 | 5 | 40 | 80 | 60 | Dead | | | |
| 4 | 5 | 29 | 55 | 60 | 8 | 2 | + | + |
| *P. chabaudi* infection controls | | | | | | | | |
| 1 | 2 | 4 | 26 | 10 | + | + | - | - |
| 2 | + | 3 | 31 | 3 | 1 | + | - | - |
| 3 | 6 | 10 | 45 | 7 | 1 | + | - | + |
| *B. rodhaini* infection controls | | | | | | | | |
| 1 | 10 | 63 | Dead | | | | | |
| 2 | 5 | 75 | Dead | | | | | |
| 3 | 3 | 7 | 40 | 60 | 73 | Dead | | |

Table 6. Percentage of parasitized erythrocytes (percent PE) in mice recovered from infection with rat strain *Plasmodium chabaudi*, after challenge with rat strain *P. chabaudi*, mouse strain *P. chabaudi* and with *Babesia rodhaini*, and the reciprocals of the titers of antibody to serum antigen (ABSA) detected with serum antigen from chickens with acute *Plasmodium gallinaceum* infection, after recovery from challenge

| Mouse No. | Days after challenge | | | | | | | | | | | | ABSA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 13 | |
| Rat *P. chabaudi* challenge % PE | | | | | | | | | | | | | |
| 1 | - | | | - | - | - | - | - | - | - | - | - | 256 |
| 2 | - | - | | - | - | - | - | - | - | - | - | - | 64 |
| 3 | - | - | | - | - | - | - | - | - | - | - | - | 16 |
| 4 | - | - | | - | - | - | - | - | - | - | - | - | 4 |
| 5 | - | - | | - | - | - | - | - | - | - | - | - | 128 |
| 6 | - | - | | - | - | - | - | - | - | - | - | - | 32 |
| 7 | - | - | | - | - | - | - | - | - | - | - | - | 128 |
| 8 | - | - | | - | - | - | - | - | - | - | - | - | 32 |
| 9 | - | - | | - | - | - | - | + | + | + | 8 | 29 | |
| Rat *P. chabaudi* challenge controls, 4 mice, average % PE | | | | | | | | | | | | | |
| | | 2.8 | | 46 | 74 | All dead | | | | | | | |
| Mouse *P. chabaudi* challenge | | | | | | | | | | | | | |
| 1 | | - | | 5 | 8 | 33 | 51 | 56 | 25 | + | + | - | 16 |
| 2 | | + | | 2 | 3 | 7 | 30 | 48 | 15 | + | - | - | - |
| 3 | | - | | 3 | 5 | 26 | 48 | 36 | 20 | + | + | - | - |
| 4 | | - | | 1 | 1 | 1 | 1 | + | + | - | - | - | 64 |
| 5 | | + | | 1 | 2 | 9 | 2 | + | + | - | - | - | 32 |

Table 6 (cont'd.)

| Mouse No. | Days after challenge 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 13 | ABSA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | | + | | 6 | 10 | 49 | 63 | 80 | 70 | Dead | | | |
| 7 | | + | | 3 | 8 | 7 | 3 | + | + | - | - | - | - |
| 8 | | + | | 3 | 8 | 6 | 7 | + | - | - | - | - | - |
| 9 | | - | | 1 | 1 | 1 | 1 | + | + | + | - | - | 8 |
| Mouse *P. chabaudi* challenge controls, 4 mice, average % PE | | | | | | | | | | | | | |
| | | .5 | | 32 | 60 | 73 | All dead | | | | | | |
| *B. rodhaini* challenge % PE | | | | | | | | | | | | | |
| 1 | | + | | + | 1 | 1 | - | - | - | - | - | - | 128 |
| 2 | | + | | 3 | 11 | 36 | 44 | 40 | 20 | + | + | - | |
| 3 | | + | | 10 | 28 | 54 | 32 | 30 | 15 | + | + | + | - |
| 4 | | + | | 10 | 22 | 56 | 63 | 42 | 7 | 3 | 3 | - | - |
| 5 | | + | | 7 | 24 | 38 | 46 | 70 | 35 | 7 | 1 | 1 | - |
| 6 | | + | | 11 | 12 | 12 | 21 | 26 | 10 | 3 | 1 | + | - |
| 7 | | 1 | | 5 | 13 | 27 | 39 | 14 | 10 | 4 | 1 | - | - |
| 8 | | + | | 17 | 34 | 55 | 64 | 36 | 7 | + | - | - | - |
| 9 | | + | | 5 | 20 | 45 | 37 | 22 | 9 | + | + | + | |
| *B. rodhaini* challenge control, 4 mice, average % PE | | | | | | | | | | | | | |
| | | .3 | | 44 | 73 | 81 | All dead | | | | | | |

and one mouse of the group died. All of the mice challenged with *B. rodhaini* developed patent parasitemia, but none of the mice died. All of the mice that had been challenged with rat strain *P. chabaudi* had ABSA in plasma samples collected at the end of the experiment. In the other groups the appearance of the antibody was variable, but tended to be found in plasma of mice that had little or no parasitemia from the challenging infections.

## Study of Parasites of a Relapsed *P. chabaudi* Infection for Antigenic Variation

As was indicated (Table 6), mouse number 9 of the group challenged with rat strain *P. chabaudi* developed patent infection on the 8th day of observation and the parasitemia reached 29% PE on the 13th day. Blood from this mouse was passed to fresh mice and the infection was maintained as a passage 'relapse' strain.

Two preliminary experiments were performed to test this strain for the antigenic variation. In each, two groups of 12 mice were infected with rat strain parasites and two other groups were given infection with the relapse strain. On the 4th day after infection, all of the mice were given latency inducing Atabrine treatment and 10 days after treatment each mouse in each group was given challenging infections of $10^5$ PE of the homologous or heterologous strain. The data on parasitemia and mortality in the two experiments are presented (Table 7). In the first experiment, 11 of 12 mice with latent rat strain infection survived challenge with the rat strain while 6 of the 12 survived challenge with the relapse strain. In mice with latent relapse strain infection, 3 of 12 survived challenge with the homologous strain and 6 of 12 survived challenge with the heterologous rat strain. Similar results were

Table 7. Summary of data on average percentage of parasitized erythrocytes (% PE) and survival from experiments in which mice with rat strain and relapse strain *Plasmodium chabaudi* were given challenge with homologous and heterologous strains of parasites after latency inducing Atabrine treatments

| | | Days after challenge | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Rat strain challenged with rat strain | % PE | + | 8.5 | 12.0 | 13.0 | 9.0 | + | - |
| | No. alive | 12 | 12 | 12 | 11 | 11 | 10 | 10 |
| Rat strain challenged with relapse strain | % PE | + | 3.0 | 20.0 | 41.0 | 21.0 | 1.5 | + |
| | No. alive | 12 | 12 | 12 | 12 | 9 | 6 | 6 |
| Relapse strain challenged with relapse strain | % PE | 3.0 | 10.0 | 23.0 | 59.0 | 18.0 | + | - |
| | No. alive | 12 | 12 | 12 | 11 | 3 | 3 | 3 |
| Relapse strain challenged with rat strain | % PE | + | 4.5 | 14.0 | 22.0 | 35.0 | + | - |
| | No. alive | 12 | 12 | 11 | 11 | 10 | 6 | 6 |

Summary of Mortality after Challenge from 2 Experiments

| | No. Chal. | No. Alive |
|---|---|---|
| Rat strain challenged with rat strain | 24 | 16 |
| Rat strain challenged with relapse strain | 24 | 7 |
| Relapse strain challenged with relapse strain | 24 | 7 |
| Relapse strain challenged with rat strain | 24 | 7 |

obtained in the second experiment and the total mortality from each challenging infection is summarized (Table 7).

## Analysis of Blood of Rats with Acute *P. chabaudi* Infection for Antigens

Plasma fractions from a Sephadex G-200 column were pooled as indicated in Figure 1. Each pool was reconstituted after lyophilization and tested for antigen activity in double diffusion in gel tests with globulin of rats hyperimmunized by *P. chabaudi* infection. Of the pooled samples tested, 3, 4 and 6 showed precipitin lines. Further tests indicated that in addition to antigen activity in pools 3, 4 and 6, pool 2 contained antibody to the antigen in pool 6 (Figure 11). While it appears that there are identical antigens in pools 3 and 4, these were not tested for identity with the antigen found in pool 6.

Since the antigen found in pool 6 appeared to be purer than those found in pools 3 and 4, it was selected for further study. As this antigen was associated with the albumin and hemoglobin fraction from the column, it was assumed to have a sedimentation coefficient of approximately 4S.

Antigen was detected with globulin of hyperimmunized rats in material leached from washed cells of malarious rat blood in double diffusion in gel tests. This antigen and pool 6 (4S) antigen were identical (Figure 12).

Samples of material processed from the cells of malarious rats, referred to as parasitic antigen, were pooled after molecular sieving in a Sephadex G-200 column as shown in Figure 2. When these pools were tested for antigen activity in double diffusion in gel tests with globulin of hyperimmunized rats, pools 1, 3 and 4 were active. Identity of the antigen of pools 3 and 4 and the 4S antigen, and material leached from washed cells, was indicated (Figure 12). The antigen found in the

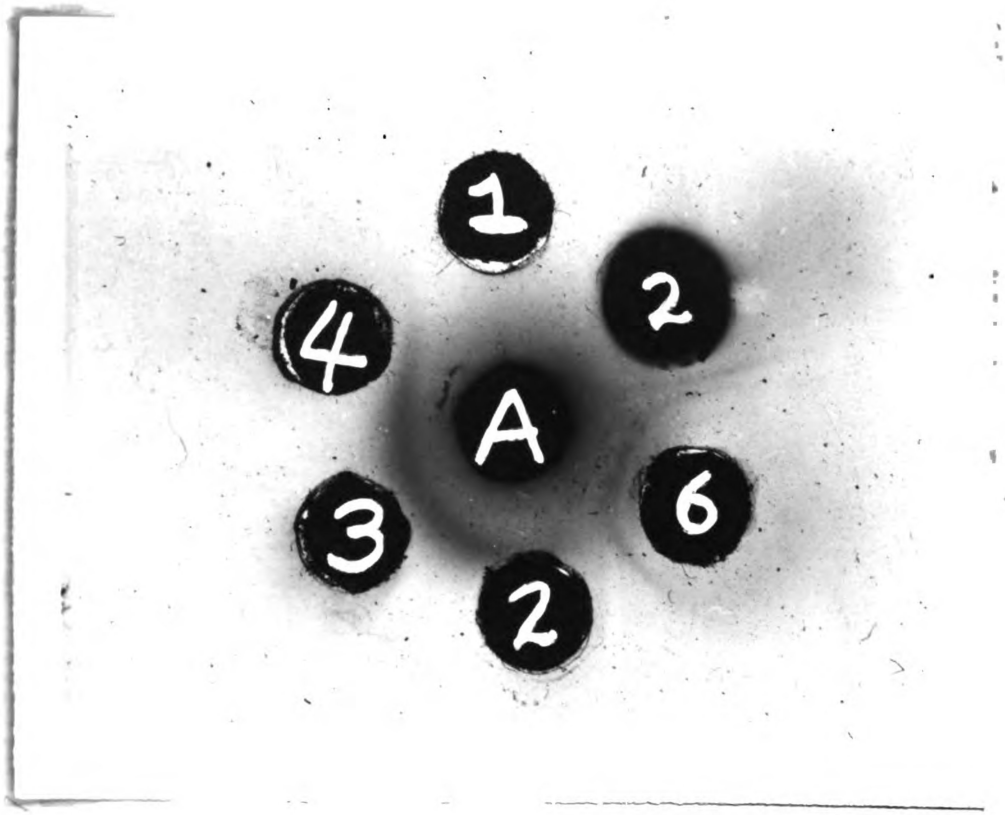



Figure 11. Tests in double diffusion in gel tests for antigen and antibody in pools of protein bearing fractions 1 through 6 of plasma of rats with acute *Plasmodium chabaudi* infection obtained after molecular sieving through a column of Sephadex G-200 as detected with antibody (A) prepared from rats hyper-immunized by *P. chabaudi* infection.


Figure 12. Reactions in double diffusion in gel test of parasitic antigen (well 1), leached material (well 2), antigen secreted in urine (well 3) and 4S antigen from plasma (well 4) with serum of rats hyperimmunized by *Plasmodium chabaudi* infection.

urine of acutely malarious rats showed precipitin lines of identity with the 4S antigen from plasma, the antigen leached from blood cells of malarious rats and the parasitic antigen (Figure 12).

The leached material was subjected to immunoelectrophoretic study along with whole normal rat serum. Tests with rabbit anti-rat serum and with guinea pig anti-rat IgG serum showed that the leached material contained a globulin fraction in the IgG class ($\gamma$1) which was not present in material leached from normal rat blood cells (Figure 13).

Tests of extracts of homogenized kidney tissue of malarious rats indicated that antigen which reacted with globulin of hyperimmunized rats was present in the supernatant buffer used in cold extraction of the homogenized tissue. This antigen also showed lines of identity with the 4S antigen from plasma. The material extracted with citrate buffer from the kidney homogenate reacted in gel tests with 4S antigen from plasma and with guinea pig anti-rat IgG ($\gamma$1) (Figure 14).

Figure 13. Immunoelectrophoretic study of material "leached" from cells of blood from rats with acute *Plasmodium chabaudi* infection. A. Reaction of normal rat serum (top well) and "leached" material (lower well) with anti-rat serum. B. Reaction of normal rat serum (top well) and "leached" material (lower well) with anti-rat IgG serum. C. Reaction of normal rat serum (top well) and material "leached" from blood cells of normal rats (lower well) with anti-rat IgG serum.

A

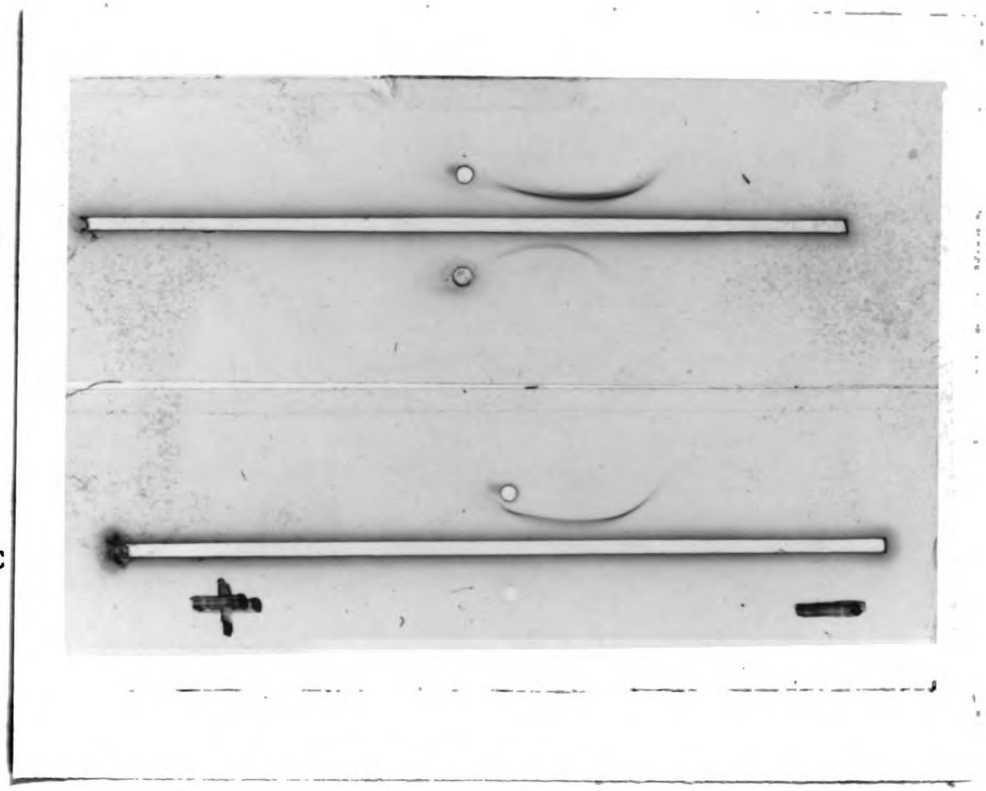



Figure 13

Figure 14. Reaction in double diffusion in gel tests of globulin extracted from kidneys of rats with acute *Plasmodium chabaudi* infections. A. Reaction between extracted globulin from kidney (well B), 4S antigen from pool 6 of plasma from blood of rats with acute *P. chabaudi* infection (well 2) and pool 6 equivalent prepared from plasma of normal rats (well 4). B. Reactions between anti-rat IgG (well A), the globulin extracted from kidney of malarious rats (well B) and extract from kidneys of normal rats (well N).

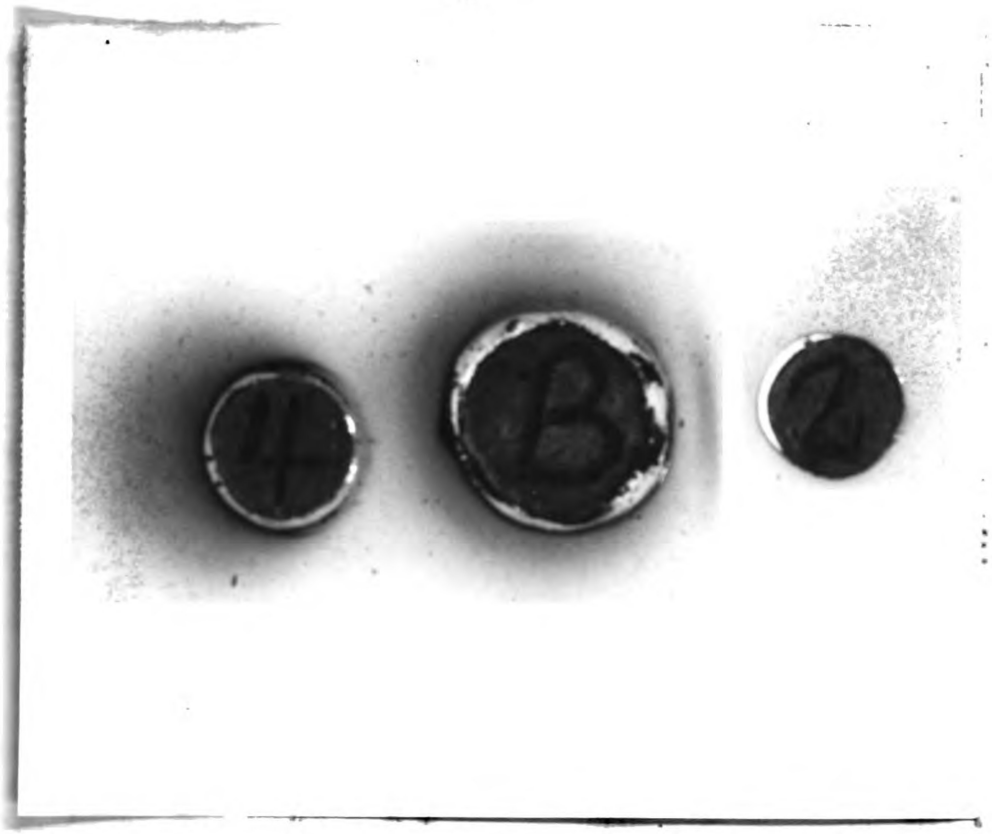



Figure 14a

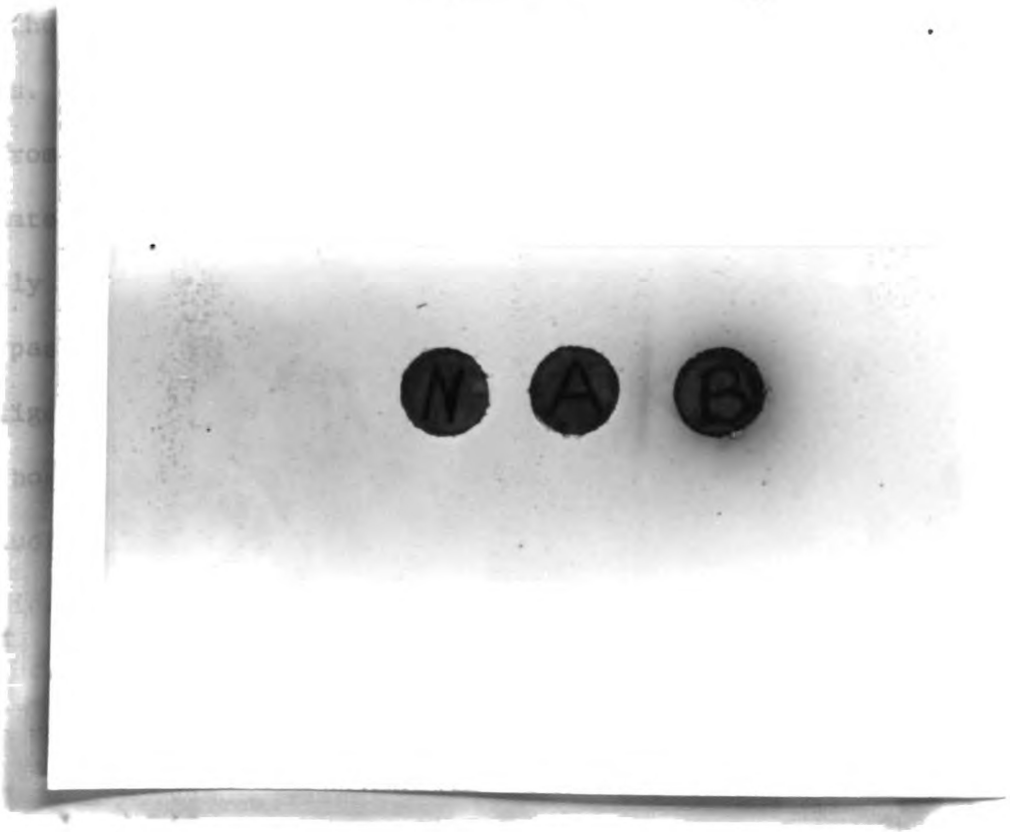
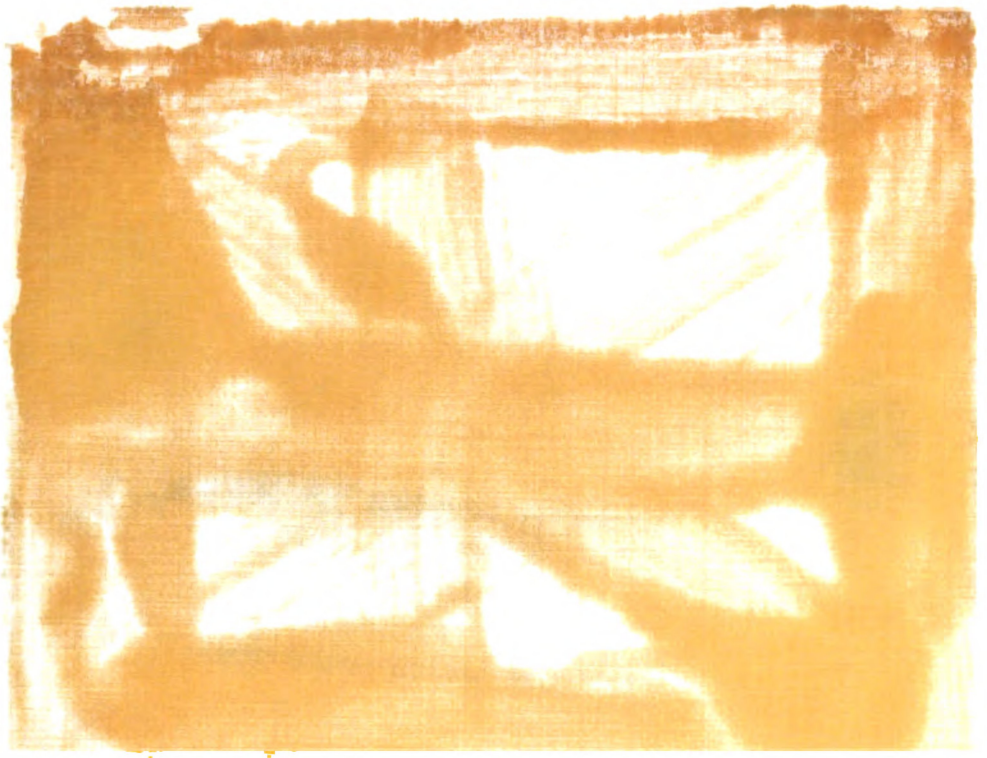



Figure 14b



# DISCUSSION

Adaptation of *P. chabaudi* to laboratory rats was achieved after some initial refractoriness during the earlier passages. Progressive adaptation was indicated by shortening of the prepatent period, lengthening of the patent period, and an increase in the percentage of parasitemia with successive passages. In splenectomized rats used for the 4th passage, parasitemia attained a level of 30% PE. However, the incompleteness of the adaptation was evident in the next passage in which rats with intact spleens were used and the parasites did no better than they had in the original passage in splenectomized animals. It was not until the 10th passage that parasitemia of 30% PE developed in intact animals.

From the data it appeared as though the immune responses which terminated parasite growth were more pronounced against parasites recently taken from the mouse host than they were to those present in later passages. It appeared that constant passage in the mouse resulted in antigenic adaptation to select parasites which were more like the mouse host and this same process was repeated when the parasite was introduced into the rat host. That this might be true was indicated when mice with induced latent rat strain infections were challenged with the rat and mouse strains. These mice exhibited a much stronger immune response to the rat strain than they did to the parasites from mice.

A significant change that seemed to have resulted from the adaptation was the loss of the immunogen which made mice recovered from *P. chabaudi* resistant to challenge with *B. rodhaini*, as reported by Cox and Milar (1968) and confirmed by Corwin and Cox (1969). While rats that had recovered from *P. chabaudi* were strongly resistant to challenge with the homologous parasite, they were no more resistant than normal rats to challenge with *B. rodhaini*. That this antigen was not associated with infection with *P. chabaudi* of rats was indicated by TBF tests using SA and ABSA from malarious and recovered chickens. Serum antigen was not found in rats with acute *P. chabaudi* infections, nor was the antibody to the antigen detected in the serum of recovered rats. Tests for these substances were negative on acutely malarious and recovered rats as late as the 35th passage. Further, in an experiment in which the rat and mice strains were compared in mice, antibody to serum antigen was not found in mice infected with the rat strain, but was present in those infected with the mouse strain on the 6th and 7th days of infection.

It appears, however, that the ability to cause serum antigen to be elaborated may in some way be determined more by the host than by the nature of the parasite. When the rat strain was reintroduced into mice and passaged several times, mice with latent rat strain *P. chabaudi* had antibodies which reacted with serum antigen from blood of chickens with acute *P. gallinaceum* infection and were as immune to *B. rodhaini* as they were to challenge with mouse strain *P. chabaudi*. Thus it was indicated that the rat strain of parasites regained the ability to cause serum antigen to be elaborated after it was returned to the mouse host.

As indicated, studies of the immunopathogenesis of *P. chabaudi* for rats were confined to parasites of the strain that had not been passed more than 20 times in the new host. In these studies there was

association of anemia with the titers of cold active agglutinin for trypsinized erythrocytes and splenomegaly just as had been reported earlier for both acute *B. rodhaini* and *P. berghei* infections of rats (Cox *et al.*, 1966; Schroeder *et al.*, 1966). The association of acute anemia with the presence of early appearing antibody to serum antigen as reported by Thoongsuwan (1971) was not evident in the present experiments.

The physical appearance of rats during the course of *P. chabaudi* infection deserves comment. Except for the pale ears and eyes, they looked remarkably healthy. They remained active and well groomed. This condition pertained even among rats of a later experiment whose acute anemia crisis was accompanied by marked hemoglobinuria. The ruffled coat, huddling and labored breathing commonly seen in mice with *P. chabaudi*, or rats with *B. rodhaini*, was not observed in rats infected for these experiments.

Study of histologic sections of kidneys of rats with acute *P. chabaudi* infection indicated that there was a glomerulonephritis associated with the infection. The onset of the nephritis, as indicated by elevations of the SKD values obtained and increases in the number of nuclei present in the glomerular tuft, became evident on the 3rd and 4th days of infection. At this point parasitemia had just begun to rise and the titers of hemagglutinin were elevated. Marked drop in RBC counts and marked splenic enlargement followed 2 days later, at which time the severity of the nephritis was maximal. Values measuring the severity of the nephritis fell after the 6th day but had not reverted to normal values on the 14th day of infection.

The changes observed in the kidney tissues were hypercellularity of glomerular tuft due to increase in number of mesangial cells, swelling of the endothelial cells of the capillary tuft and swelling

of the epithelial cells of the convoluted tubules. The presence of hyaline casts indicated that the changes were accompanied by considerable extravasation of serum proteins. This cellular swelling appeared to close the lumen of the capillaries and the convoluted tubules. On the 5th and 6th days of infection, the extent of the swelling suggested that kidney function may have been reduced temporarily.

Sections of kidneys from rats taken later in the course of infection indicated that a less acute condition pertained. The glomeruli were nearer normal size but were frequently lobated and fibrinous adherence to Bowman's membrane was often observed. Tubular elements of the nephron did not appear to have regenerated after the initial change. Extensive desquamation of the lumen epithelium, along with a thickened basement membrane of the tubule, remained a common finding (Figure 3c).

A study of the materials passed with the urine indicated that the severity of kidney damage may have been greater than was indicated by histopathologic study. Proteinuria rose sharply after the 3rd day of infection from a normal of about 1 mg./ml. of urine to 42 mg. on the 7th day of infection. Thereafter protein concentration fell gradually to about 20 mg. on the 13th day but thereafter remained stable for more than the 22 days of observation. Analysis of the protein found at the peak of output indicated that there were at least 5 serum components present including albumin and IgG ($\gamma$1 fraction).

Also present from the 4th through the 7th day was a marked hemoglobinuria with the urine being of the color of dark port wine on the 5th through the 6th day. The urine collected on the 9th day was of normal color; however, the slow recovery from the anemia suggested that destruction of blood elements may have continued. Red blood cell counts made on the 22nd day of the experiment remained lower than normal.

An analysis of antigens associated with acute malarial infections was undertaken to attempt to identify antigens associated with immunopathologic processes of acute *P. chabaudi* infection. The study of malarious blood indicated the presence of antigens which reacted with the serum of hyperimmunized rats in plasma material leached from the washed cells of infected blood and from the parasite fraction obtained from saponin treated cells. Of these blood fractions the plasma appeared to be richer in antigenic components than the others. Antigenic activity was obtained with plasma fractions 3, 4 and 6 obtained by subjecting the plasma to column chromatography (Figure 1). Of these active fractions the 6th was distinctly separated from other antigens in plasma and appeared to be a single antigenic component. Since this antigen had been eluted with the albumin-hemoglobin fraction, it was assumed to have a sedimentation coefficient of 4S and was designated as 4S antigen. In double diffusion in gel tests with globulin of hyperimmunized rats, the 4S antigen line showed identity with material leached from washed cells of malarious blood and the purified parasite fraction obtained after saponin treatment of cells from malarious blood. Tests of the 4S antigen with the globulin of rats immunized with the plasma of chickens with acute *P. gallinaceum* and of rats immunized with plasma of rats with acute *B. rodhaini* suggested that it was an antigen exclusively associated with the *P. chabaudi* parasite.

Tests of extracts of homogenates of the kidneys of malarious rats and of the protein secreted in their urine, indicated that in both materials there was antigen present which reacted with the globulin of hyperimmunized rats. When these antigens, extracted from these materials, were tested in gel along with the 4S antigen, lines of identity indicated that the 4S antigen was present in the kidney tissues and was

secreted in the urine from the 4th through the 7th day of infection.

The 4S antigen was used to examine plasma of malarious rats. Precipitin lines resulted which indicated that antibody to the antigen was present along with antigen during the acute malaria period. That the 4S antigen and its antibody were readily dissociable was further indicated by the finding that the antibody was present in the 2nd and the antigen in the 6th fractions obtained from the Sephadex G-200 column. That is, antibody to 4S antigen separated from the plasma along with the IgG class of globulins, while the antigen was passed with the albumin-hemoglobin fraction. The presence of 4S antigen in the material leached from cells of infected blood, in kidney tissue homogenate and in the urine, along with the presence of IgG, suggests that complexes of the antigen, and its antibody, bound to blood cells and kidney tissue were also readily dissociated.

In the experiments where the relapsed infection of rat strain *P. chabaudi* was tested for evidence of antigenic variation, it was clear that mice with latent rat strain infection were much more resistant to challenge with the homologous strain of parasites than they were to the relapse strain. The data also indicated that mice given latency inducing treatments after infection with the relapse strain of parasites were no more immune to the relapse strain than they were to the rat strain. In the 2 experiments 18 of 24 mice with latent rat strain infection survived challenge with the homologous strain while only 7 of 24 survived challenge with the relapse parasites. Mice with latent relapse strain infections had only 7 of 24 mice that recovered from homologous challenge and 7 of 24 that survived challenge with the rat strain. The data suggested that rather than antigenic variation, in which the relapse parasites acquired antigens not expressed by the parent strain, as was

demonstrated in the relapse of *Borellia recurrentis* by Meleney (1928a, 1928b), the relapse strain of *P. chabaudi* seemed to have lost immunogenic properties. These findings are similar to those obtained in a study of relapse mechanisms of *P. berghei* in mice in which the data obtained led to essentially the same conclusion (Cox, 1959, 1962).

Past study has emphasized the roles of antigens and antibodies that seem unrelated to parasite species in the pathogenesis and acquired immunity of red blood cell infections. Cox *et al.* (1966) and Schroeder *et al.* (1966) related the appearance of cold active agglutinin for trypsinized erythrocytes to massive blood loss, splenic enlargement and erythrophagocytosis in acute malaria and babesiosis of rodents and suggested that the mechanism of red blood cell destruction might be instrumental in the recovery from acute infection. Similar cold agglutinins have been associated with anemia, splenomegaly and erythrophagocytosis in other infectious anemias, e.g., anaplasmosis, eperythrozoonosis, hemobartonellosis and equine infectious anemia (Schroeder *et al.*, 1965; Cox and Iturri, 1973; Oki and Miura, 1970). The present work shows the presence of the same type of agglutinin associated with anemia of acute *P. chabaudi* infections of rats. However, none of the work cited has clearly indicated a role for the agglutinin in blood loss during acute red blood cell infections.

Blood loss in normal rats was observed to follow the injection of globulin of monkeys with acute *P. knowlesi*, and injection of globulin of either rats or dogs with acute babesiosis produced anemia in normal rats and dogs. However, the mechanisms responsible for this blood loss were not clear since serum antigen was the only substance recognized at the time (Cox, 1966; Sibinovic *et al.*, 1967a, 1967b, 1969; Corwin and Cox, 1969; Cox and Iturri, 1973).

It was not until recently that it was found that antibody to serum antigen appears soon after the antigen and that for several days thereafter both the antigen and antibody can be readily demonstrated in the serum. It was further indicated that anemia did not become a prominent feature of the infection until the antibody was detected (Thoongsuwan, 1971). This concurrent presence of antigen and antibody indicates that these substances are readily dissociable. The TBF tests for these antigens and antibodies indicated that they are cold active substances since the tests worked more effectively between 5-20 C and not at all at temperatures above 28 C. *In vivo* such antigens and antibodies may form complexes and bind complement and as such react with red cells to act as opsonins or even cause hemolysis (Dixon, 1966; Unanue and Dixon, 1968; Dixon, 1971). Further, complexes of antigen, antibody and complement can affect vascular permeability causing vascular leakage and may also act as immunotoxins (Ward, 1970).

In view of this newer knowledge it now seems proper to suggest that the cold active agglutinin for trypsinized cells may not be solely responsible for anemia and nephritis as was suggested (Cox *et al.*, 1966; Schroeder *et al.*, 1966; Iturri and Cox, 1969). While this agglutinin may have been in part a causal mechanism, a role for complexes of serum antigen, antibody and complement must be given serious consideration.

However, in the present work it was shown that while serum antigen and its antibody were associated with acute *P. chabaudi* infections of mice, they were not detected in any of the rats with acute *P. chabaudi* infection. It was therefore concluded that complexes of serum antigen and its antibody were not involved in the blood loss and nephritis observed in this work. However, dissociated 4S antigen and its antibody

were present in the plasma of rats with acute *P. chabaudi* infection and it is suggested that complexes of this antigen and its antibody could have accounted for the observed anemia and nephritis.

It is also pointed out that in the present work only one of several antigenic components of the blood of malarious rats was studied and shown to stimulate low avidity antibody. Other antigenic substances, such as those found in plasma fraction pools 3 and 4, may act in a similar manner and also contribute to the pathogenic mechanisms.

It would appear probable that other red blood cell parasites would also have antigens that were unique to the parasite which might stimulate production of low avidity antibody. A role for such antigens in pathogenesis of infections other than *P. chabaudi* of rats should therefore be considered. Further, in those red blood cell infections in which serum antigen is elaborated, the effects on pathogenesis should be additive. The nephritis observed in the present experiments was similar to, but not as severe as, that observed in acute *B. rodhaini* infections of rats by Iturri and Cox (1969). Since serum antigen was elaborated during *B. rodhaini* infections, it might be assumed that this difference was quantitative.

Inasmuch as the 4S antigen and its antibody were present in the plasma, leached from cells of malarious rat blood, eluted from the homogenized kidney tissues of malarious rats and also present in the urine of infected rats at the time of development of anemia and nephritis, it is clearly possible that they could have had a role in pathogenesis. Further, the indication that the 4S antigen was exclusively an antigen of *P. chabaudi* suggested that in the present study the immunopathogenic substances involved in anemia and nephritis were in part parasite mediated.

## SUMMARY

This research indicated that *P. chabaudi* infections of rats differed from those of mice in that serum antigen responsible for immunity to *B. rodhaini* was not elaborated in rat infections.

In rats the infection caused anemia with splenomegaly, intravascular hemolysis and hemoglobinuria. An acute glomerulonephritis with marked proteinuria was also associated with the anemia crisis. It appeared that there was residual kidney damage from the acute nephritis since proteinuria was a constant finding for more than 30 days after recovery.

Studies of the blood plasma of malarious rats indicated the presence of a soluble antigen and simultaneously, antibody to the antigen. The ease with which both antigen and antibody reactions could be demonstrated, and the ease with which they could be separated by column chromatography suggested that the antibody had a low avidity for the antigen. Both the antigen and its antibody were present in material leached from washed cells of infected blood, were extracted from washed kidney tissue homogenates, and were present in the urine of rats at the peak of malarial infections.

It is suggested that the low avidity antibody and the soluble antigen formed complexes which, in addition to reacting with the surface of erythrocytes to act as opsonin, may also fix complement to cause hemolysis, increase vascular permeability and act as immunotoxins.

It is suggested further that these complexes of antibody and soluble antigen may have a greater role in blood loss and nephritis in acute malaria than does the parasite or direct action of antibody with parasites.

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# APPENDICES

## APPENDIX A

Double Diffusion Gel Test

Buffer:

Barbital ionicity 0.15 pH 7.4

6.98 gm. sodium barbital

6.0 gm. sodium chloride

2.7 ml. in hydrochloric acid

## APPENDIX B

Amidoschwarz (Uriel, 1958)

0.1 qm. amidoschwarz

45 ml. 12% acetic acid

45 ml. 1.6% sodium acetate

10 ml. glycerol

Differentiate in 2% acetic acid

# APPENDIX C

## Disc Electrophoresis

**Buffers:**

A. 6 gm. TRIS
0.8 ml. conc. HCl } 100 ml. dist. $H_2O$

B. 0.75 g. TRIS
0.4 ml. conc. HCl } 100 ml. dist. $H_2O$

C. 0.6 g. TRIS
3 glycine - 2 liters dist. $H_2O$

**Running Gel:**

1.4 gm. cyanogum for gel - 7% or 0.9 gm. for 4.5% gel

20 ml. buffer A

0.02 ml. TEMED

0.02 gm. Ammonium Persulfate

**Sample Gel:**

4 gm. acrylamide

5 gm. sucrose

100 ml. buffer B

0.1 gm. Ap. 0.1 ml. TEMED

**Spacer Gel:**

0.4 g. cyanogum

in 10 ml. buffer B

0.1 ml. TEMED

0.01 gm. Ammonium Persulfate

## APPENDIX D

O. IM Phosphate Buffer pH 7.5

Solution A: $NaH_2PO_4 \cdot H_2O$, 27.6 gm./liter

Solution B: $Na_2HPO_4$, 28.4 gm./liter

Solution

A - 80 ml.

B - 420 ml.

make up to 1 liter, and add 11.7 gm. of NaCl

## APPENDIX E

TRIS-HCl 0.2 NaCl pH 8.0

TRIS 0.1 M (12.11 gm./liter)

NaCl 0.2 M (11.69 gm./liter)

Dissolve in 2/3 of buffer volume with deionized distilled water

Add 1 N HCl to pH 8.0

Check pH as final volume is approached.