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CHARACTERIZATION OF THE ANTIGENS OF PLASMODIUM BERGHEI

The Ohio State University

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CHARACTERIZATION OF THE ANTIGENS OF

PLASMODIUM BERGHEI

DISSERTATION

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

By

Gary David Grotnaus, B.S., M.Sc.

* * * * *

The Ohio State University
1982

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FIELDS OF STUDY

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INTRODUCTION

The malaria parasite is enormously complex antigenically and it would be desirable to identify specific antigenic components which are capable of inducing a truly protective immune response. Some antigens probably have no protection stimulating characteristics while others may even initiate responses which are harmful to the host. Very little work relating to the identification and characterization of plasmodial components using modern biochemical separation techniques has been attempted. Even fewer attempts have been made to correlate immunogenicity with the biochemical characteristics of plasmodial fractions.

In this dissertation, we report the characterization of some of the components of P. berghei using polyacrylamide gel electrophoresis metabolic labelling and immunoprecipitation. These studies show that the immunity inducing component of the plasmodia exists bound to the membrane and also in soluble form. We determined which of the components are present in the membranes and which are present in the soluble fractions. A very high level of protection was stimulated by vaccinating rats with material washed from the surface of free plasmodia. We characterized the immune response of rats to infection and further studied the composition of the plasmodial antigens reacting with the rats serum by immunoprecipitation of radiolabelled antigens. From the results of these studies, we developed the hypothesis that the
protection-stimulating surface coat component can exist in two forms. The form bound to the membrane is insoluble and has a high molecular weight. The other form which can be cleared from the membrane, has a lower molecular weight and consists of the hydrophilic portion of the larger membrane bound molecule.
A. LIFE CYCLE AND EPIDEMIOLOGY

The biological system of malaria encompasses various plasmodial species, their vertebrate and arthropod hosts, and the physical environment of the hosts. *Plasmodium species* are members of the suborder *Haemosporina* within the subphylum *Sporozoa*. They are classified among this group of protozoans because they undergo both a cycle of sexual reproduction and a cycle of asexual schizogony (Baker, 1969). Similarities in life cycles and general morphology have led taxonomists to believe that the family of malaria parasites may be evolutionary descendants of the coccidians of vertebrates (Levine, 1961).

Plasmodia are obligate intracellular parasites for a majority of their life cycle. They have two hosts: an invertebrate host, in which the cycle of sexual reproduction occurs, and the vertebrate host in which the cycles of asexual reproduction or schizogony occur. The Anophelene or Culicine mosquito is the vector for all known species of plasmodia.

There are over 100 known species of *plasmodia*. Plasmodia parasitize a variety of vertebrates, including humans and the higher primates, birds and reptiles. Mosquitoes capable of transmitting malaria inhabit nearly all tropical and warm climates, and once infected, they remain infected for life. Malaria is therefore endemic wherever there is contact between the parasites, the vectors and susceptible hosts.
The vertebrate host becomes infected when an infected mosquito, the vector, injects the sporozoite stage of the parasite along with an anticoagulant secretion while taking a blood meal. The sporozoites are injected directly into the capillaries from the salivary glands of the female mosquito. The sporozoites move quickly through the blood stream to eventually enter a "fixed tissue" of the vertebrate. This "fixed tissue" is the lymphoid-macrophage tissue in avian or reptilian hosts or the liver parenchyma in mammalian hosts. The blood is temporarily free of parasites while the sporozoites penetrate fixed tissues and initiate primary exoerythrocytic schizogony. After penetration of the fixed tissue, the parasite nucleus enlarges and divides by multiple fission until the cell is teeming with parasites. The invaded cell finally bursts, releasing large numbers of parasites, now called merozoites, into the bloodstream. Depending on the species of plasmodia, some merozoites may reenter the fixed tissues to complete additional exoerythrocytic cycles.

Most of the released merozoites, however, enter the erythrocytes of the vertebrate host and differentiate into either sexual or asexual forms. In the erythrocyte, the cycle of asexual schizogony begins. The parasite grows and the nucleus divides by multiple fission until a schizont is produced which is capable of releasing 8 to 16 merozoites, then the cycle is repeated. The merozoites which are destined to become sexual or gametocyte forms also enter a host erythrocyte, but they do not begin a cycle of erythrocytic schizogony. The gametocyte which develops is capable of completing its cycle of sexual reproduction only if it is ingested with the blood meal of a mosquito.
If erythrocytes containing gametes do enter the mosquito's gut, they differentiate into male or female forms, which are called microgametes and macrogametes, respectively. The microgamete and the macrogamete fuse to form a motile zygote called an ookinete which passes through the outer wall of the mosquito gut and into the haemocoel. The ookinete matures and develops into a large, tumor-like mass called the oocyst which may release over 10,000 sporozoites, some of which eventually relocate in the salivary glands of the mosquito and are capable of completing the cycle through the infection of a vertebrate host.

In mammals, much of the pathology of the disease is associated with the erythrocytic cycle of schizogony, during which large numbers of red blood cells are ruptured, releasing waste products of parasite metabolism and pigment as well as erythrocyte fragments and parasites into the blood stream. The toxicity of some or all of these products, or more likely the consequences of their reaction with antibody and the associated reduction in number of functional erythrocytes, cause the paroxysms of severe chills and burning fever characteristic of malaria. The pathology of the disease also includes enlargement of the spleen (splenomegaly) and of the liver (hepatomegaly), due to massive increases in the number of cells in the lymphoid system. Death usually results from acute anemia, although falciparum malaria may cause death in some instances by clogging small capillaries in the brain with agglutinated, infected erythrocytes (Edington and Giles, 1974).

Although malaria has largely been absent from the United States since 1951, this disease still causes much loss of life and
debilitation throughout the world (Wernsdorfer, 1977). A large majority of the world's tropical regions have environmental conditions which are optimal for malaria. A special edition of The Morbidity and Mortality Weekly Report dealing with the subject of malaria (March 10, 1978) listed 128 countries as potentially hazardous due to risk of infection with plasmodia. Areas where malaria is presently known to be a major health problem include parts of Mexico, Haiti, Central America, South America, Africa, the Middle East, the Indian Subcontinent, Southeast Asia, Korea, and Oceana.

Considering the widespread geographical distribution, it is easy to see why malaria is still a major cause of death on a world-wide scale. Nearly 800 million people (39% of whom once lived in malarious areas) now live in areas which are malaria-free, but another 800 million persons still live in areas which have absolutely no specific malaria control programs under application (Lepes, 1977). India alone was estimated to have between 4 and 5 million cases of malaria in 1974 (Jeffry, 1976). In Africa, each person receives between 40 and 120 bites yearly from infected mosquitoes, resulting in an annual total of 96 million cases and at least 1 million deaths from malaria (Peters, 1972).

Even in the United States, the incidence of malaria rose from 62 cases in 1960 to 4247 cases in 1970. This large increase was probably due to the return of infected United States military personnel from the conflict in Viet Nam. The number of American soldiers evacuated from Southeast Asia due to infection with malaria was equal to the number evacuated due to wounds (Canfield, 1972; Tigertt, 1966). In 1968,
United States forces in Viet Nam had 17,000 cases of malaria at a medical cost of about 11 million dollars (Gochenour, 1969).

B. VECTOR ERADICATION

The discovery that insecticides with residual effect can interrupt the transmission of malaria is primarily responsible for the concept of vector eradication (Lepes, 1972). Early successes led to high optimism, and it was commonly believed that malaria could be eliminated within a few years by eradication of the vector (Jeffry, 1976). In many areas vector eradication programs have failed mainly due to the fact that many mosquitoes have developed a resistance to dieldrind and DDT. Resistance to these commonly used insecticides has reduced the effectiveness of spraying to a considerable degree (Peters, 1972). Pressure from environmental groups for the use of non-toxic compounds, lack of adequate and timely financial support, and resistance from uneducated and primitive tribes have all been cited as reasons for the failure of eradication programs.

C. CHEMOTHERAPY

The field of chemotherapy, like vector eradication, also appeared to have great potential in the battle against malaria. At the time of the Korean War, through the use of primaquine and chloroquine, malaria was one of the few known diseases for which there was available specific and reasonably effective chemotherapeutic agents. Since that time, developments in the field of chemotherapy have been slow and the drug industry has failed to produce new compounds which are truly
effective in the prevention of malaria or treatment of malaria.

Since 1900, almost 200,000 drugs have been screened in the United States alone, without revealing many promising compounds (W.H.O., 1968), although some antimalarial compounds have shown promise in animal systems (Schmidt, 1973). One of the problems has been the failure to develop a tissue schizonticide which acts upon exoerythrocytic forms to effect a total cure. Strict limitations on the use of humans for drug testing and levels of tolerable side effects set by food and drug control agencies have also reduced the number of marketable new compounds. Reports of parasites which had developed resistance to chloroquine began around 1960, and high levels of chloroquine resistance are now common in parasites in parts of Southern Africa and Southern Asia (Peters, 1970).

The attempts to totally eliminate malaria by combining programs of vector eradication and treatment by chemotherapy have failed. The goal of total elimination of malaria may have been too optimistic from the beginning. However, persistent application of these programs has undoubtedly contributed to the degree of control over malaria which we do possess in the world today. Many battles against malaria have been won on the local level, and the general benefits derived from these programs have certainly lessened the degree of human suffering in many areas of the world.

D. IMMUNIZATION

At the present time, malaria is still a major health problem, and there is growing interest in developing malaria control in the field by
application of techniques based in immunology (W.H.O., 1975). Studies relating to immunization and other aspects of what is now the field of malaria immunology began at about the same time as the vector eradication and chemotherapy programs.

One of the earliest attempts at immunization was made by Sergent and Sergent (1910). They attempted to actively immunize monkeys and birds by injecting killed or attenuated sporozoites obtained from infected mosquitoes. There were many vaccination trials reported during the 1930's and 1940's after the pioneering work by Mulligan and Sinton (1933) and Freund, et al. (1945a, 1945b, 1948). These workers reported partially successful immunizations of ducks and monkeys.

During the past twenty years, multitudes of vaccinations using various host-parasite systems and life cycle stages of the parasite have been attempted.

1. Exoerythrocytic merozoites. Graham et al. (1973) attempted to immunize turkeys by chloroquine cure after they had been infected with Plasmodium falciparum exoerythrocytic forms. Although many of these turkeys died, passive transfer of the serum from the surviving turkeys protected other turkeys from exoerythrocytic challenge. Holbrook, et al. (1974) immunized with formalin-killed exoerythrocytic merozoites plus Freund's complete adjuvant, also using the Plasmodium falciparum-turkey system. They obtained some protection from exoerythrocytic challenge but later determined that the exoerythrocytic vaccination did not protect against blood stage challenge (Holbrook, et al., 1976). Recently, exoerythrocytic merozoites of Plasmodium lophurae have been purified by ion-exchange chromatography (Hollingdale
and Kilijian, 1979). This procedure may be useful in future preparation of large volumes of purified exoerythrocytic merozoites for vaccination purposes.

2. Gametocytes. Another approach to vaccination has been to induce immunity against the parasite stages which infect the mosquito and block transmission by the vector. Formalin-treated or X-irradiated gamete vaccines have been used to block transmission of malaria by mosquitoes which had been fed on chickens immunized with *Plasmodium gallinaceum* gametes (Gwadz, 1976; Carter and Chin, 1976). Immunization with gametes may limit transmission, but does not give direct protection against blood from infection.

3. Sporozoites. There have been a large number of attempts to induce immunity through the use of killed or attenuated sporozoites. Richards (1966) immunized chickens with three injections of dried or UV attenuated *Plasmodium gallinaceum* sporozoites. This same procedure was adapted to the *Plasmodium berghei* system by Nussenzwieg, et al. (1967, 1969). Killed sporozoite vaccines were not as effective as those which were attenuated by ultraviolet irradiation. Variations in the immunizing dose (Nussenzwieg, Vanderberg and Most, 1969), challenge dose (Nussenzwieg, Sanabria and Most, 1972), duration of protection (Vanderberg, Nussenzwieg and Most, 1970) and route of inoculation (Spitalny and Nussenzwieg, 1972; Spitalny, 1973) have all been evaluated within this system.

Clyde et al. (1975) have reported the immunization of human volunteers against *Plasmodium falciparum* and *Plasmodium vivax* malarias by exposing the humans to the bites of large numbers of irradiated
mosquitoes. Heat or formalin-killed sporozoites (Alger and Harant, 1976a) and freeze thawed sporozoites (Alger and Harant, 1976b) have also been shown to induce low levels of immunity to Plasmodium berghei in mice.

The practicality of sporozoite vaccination is limited by the large number of sporozoites required and the number of immunizing injections required. Immunization with sporozoites does not generally confer immunity to challenge with blood stage parasites and the ability of sporozoite vaccines to protect from heterologous sporozoite challenge is also rather limited (Richards, 1977).

4. Mixed Erythrocytic Free Parasites and Infected Erythrocytes. There have been many vaccination attempts using mixed populations of asexual blood parasites or infected erythrocytes as antigens. In the 1960's, Corradetti et al. (1966) vaccinated with x-irradiated Plasmodium berghei blood parasites. They reported partial protection of rodents against what would have been a lethal challenge. Zuckerman et al. (1967) vaccinated weanling rats with an extract of ground Plasmodium berghei blood forms. Other vaccines consisting of x-irradiated mixed blood forms protected mice and rats from normally fatal challenge doses of Plasmodium berghei blood parasites (Wellde et al., 1969; Sadun et al., 1969). Other workers, using mixed free parasites or extracts from the blood of rodents (D'Antonio, 1972; Simpson, et al., 1974) and monkeys (Brown, 1970; Schenkel, 1973; and D'Antonio, 1974) have reported varying degrees of success with their systems.
Methods used for obtaining erythrocytic free parasites and infected red blood cells used in the above studies have been widely variable and at least somewhat controversial. Procedures used to release malaria parasites or antigens from infected host cells include hypotonic lysis (Chavin, 1966; Coggshall and Eaton, 1938; Stein and Desowitz, 1964), freeze-thawing (Sadun and Gore, 1968; Mathews, et al., 1975), lysis with saponin (Spira and Zuckerman, 1962; Zuckerman, et al., 1967), lysis with ammonium chloride (Martin, et al., 1971), lysis using antiserum (Trager, et al., 1972), disruption with a french pressure cell (D'Antonio, et al., 1966a, D'Antonio, et al., 1966b), lysis by ultrasound (Prior and Kreier, 1972; Prior and Kreier, 1977), and natural release (Dennis, et al., 1975; Mitchell, et al., 1974; Trager and Jensen, 1976).

Most free parasite preparations contain at least some host cell contamination which can be detected by examination using electron microscopy (Aikawa and Cook, 1971). A review of the various techniques used for the isolation of parasites from red blood cells has been prepared by Kreier (1977). Kreier concluded that all procedures yielded antigen preparations which were suitable for some purposes, but that only natural release of parasites in culture and continuous-flow sonication provide large quantities of morphologically intact parasites. There have also been a number of solubilization procedures used to prepare antigens from free parasites or infected erythrocytes for use as vaccines or serological antigens. These procedures have been reviewed by Grothaus (M.Sc. Thesis, The Ohio State University, 1978).
It is also noteworthy that nearly all of the successful erythrocytic vaccinations reported have included adjuvants in the vaccines. Freund's complete adjuvant has been the successful in these systems. Saponin, hexylamine, *Bordetella pertussis* vaccine, bacterial endotoxin, levamisole and vitamin A have also been used as adjuvants with malaria vaccines. Many of these adjuvants have been evaluated when injected with a soluble *Plasmodium berghei* antigen by Desowitz (1975).

5. *Merozoite and Late Stage*. Recent work has indicated that an important part of the immune response of the host is probably directed against the erythrocytic merozoite. The morphology of the merozoite has been studied by Aikawa (1977; 1971). Merozoites are highly differentiated forms of the parasite with a specific structure. They contain a thick membrane inside the plasmalemma, a conically shaped anterior, and organelles which include a cytostome, a mitochondrial, a nucleus, endoplasmic reticulum and ribosomes. They also possess a set of special organelles called rhoptries and micronemes which are associated with the process of erythrocyte penetration. This form of the parasite, the merozoite, is exposed to the mechanisms of the host's immune response as it passes from erythrocyte to erythrocyte in the blood stream. The entire merozoite is covered with a surface coat, i.e., capsule, which may play an important role in the evasion of the host's immune response by the parasite.

Vaccinations with blood forms enriched in schizonts and merozoites, or more recently, with preparations consisting almost
entirely of merozoites, have shown both a higher degree and broader spectrum of protection than any other vaccinations.

One of the first immunizations which achieved substantial protection in monkeys was performed with an antigen consisting of a selected population of killed *Plasmodium knowlesi* schizonts plus Freund's complete adjuvant (K.N. Brown, 1968). These monkeys showed a prolonged latent period after challenge and eventually eliminated their parasitemias. Voller and Richards (1969), using a similar preparation of *Plasmodium falciparum* schizonts, found that immunized animals had a longer prepatent period, although they eventually died. Brown and Tanaka (1975) vaccinated monkeys against *Plasmodium knowlesi* malaria using a schizont antigen plus Freund's complete adjuvant. In these experiments, eight of thirteen monkeys survived what would normally have been a lethal challenge dose.

Another partially purified late stage antigen was prepared by Siddiqui, et al. (1977). Erythrocytes containing segmenters, schizonts and mature trophozoite stages of parasites were collected from culture. These infected erythrocytes were lysed with saponin and partially purified by density gradient centrifugation. Three of three owl monkeys which had been vaccinated with this preparation survived what would have been lethal challenge with a homologous variant. Two of these same monkeys were later shown to be protected against subsequent challenge with a heterologous strain (Siddiqui, et al., 1978). These results were particularly significant in that they showed a broader spectrum of protection than that shown in any previously reported work.
Studies indicating that antibody capable of inhibiting erythrocyte reinvasion by merozoites could be correlated with protective immunity were reported using *Plasmodium falciparum* from the blood of infected Gambians (Cohen, *et al.*, 1961) and *Plasmodium knowlesi* from culture (Cohen, *et al.*, 1969). It was this work which first stimulated efforts to isolate the merozoite stage of the parasite for use as an antigen.

Incubation of schizonts in culture medium at 37°C in suitable flasks under a 5% CO₂ atmosphere resulted in rupture of mature segmenters and the eventual release of free merozoites. The addition of phytohemagglutinin (Mitchell, *et al.*, 1975) or schizont agglutinating antiserum (Mitchell, *et al.*, 1973) caused clumping of the remaining schizonts. These clumped cells could then be removed by low speed centrifugation, leaving a supernatant rich in merozoites.

A less complicated method which yields an even larger and cleaner preparation of merozoites has been introduced by Dennis, *et al.* (1975). This method consists of culturing infected red blood cells in a special chamber which is fitted with a polycarbonate sieve. When merozoites are released from a mature schizont, they pass through the 2 um pores of the sieve into another chamber where they are collected. Intact erythrocytes are too large to pass through the holes, so they are retained in the original chamber. Another technique for collection of merozoites uses the principle that concanavalin A-sepharose columns are capable of retaining infected erythrocytes, but not free merozoites. Infected red blood cells are bound to the column, incubated under natural conditions (37°C, CO₂, etc.) and allowed to mature. Merozoites are eventually released and washed out of the column with culture
medium, and collected. This system has been used to produce pure populations of *Plasmodium chabaudi* (David, et al., 1978) and *Plasmodium falciparum* (Billiault and Ambrose-Thomas, 1980) merozoites.

A majority of the immunizations using merozoites have been done at Guy's Hospital Medical School (Mitchell, et al., 1975; 1977a; 1977b). Monkeys were immunized with either freshly prepared merozoites or merozoites which were stored at -70 °C or freeze-dried merozoites stored at 4 °C. The vaccines were prepared by mixing between $5 \times 10^6$ and $5 \times 10^9$ merozoites or equivalent amounts of freeze-dried material with equal volumes of Freund's complete adjuvant. Two or three intramuscular injections were given over periods ranging from 22 to 98 days. All monkeys (7/7) survived homologous blood stage challenge (two without patent infection). Challenge with heterologous blood stage parasites killed only one of twelve monkeys while two animals from this group were parasitologically negative. Sporozoite-induced challenges were similarly cleared in 2/2 monkeys (Richards, 1977). Collins, et al. (1979), using the *Plasmodium fragile - Macaca mulatta* system have been successful in protecting 3 of 4 monkeys from a normally lethal challenge after vaccination with a nearly identical merozoite antigen.

Immunization of rodents against *Plasmodium berghei* using a population of sonically-freed plasmodia rich in merozoites was also successful (Saul and Kreier, 1977). A variety of types of adjuvant, the quantity of the immunizing dose and various antigen preparations were tested in this study.

6. Summary of Immunizations. Immunizations with exoerythrocytic merozoites, gametocytes, sporozoites or mixed free parasites have not
been entirely satisfactory. Partial protection has been obtained from vaccinations using these types of plasmodial preparations. However, in many cases the degree of protection obtained has been minimal. Practical limitations to the use of these vaccines include the inability of these vaccines to protect against challenge with stages of the parasite other than those used for immunization or even intrastrain variants of the same life stage. The isolation and purification of sufficient numbers of parasites for immunization still remains a problem in the use of these systems.

Vaccinations have been more successful using late stage antigens consisting of schizonts, segmenters and mature merozoites than vaccinations using only trophozoites. This discovery influenced researchers to develop techniques for the isolation and purification of large numbers of pure merozoites. As stated earlier, the pure merozoite immunizations have shown a higher degree and broader spectrum of protection than have any other immunizations to date.

E. CHARACTERIZATION OF PLASMODIAL ANTIGENS

A majority of the work relating to the antigenic characterization of plasmodial fractions has related to their reactivity in various serodiagnostic testing procedures. In most early procedures, the free parasites or infected erythrocytes were simply solubilized by physical procedures such as hypotonic lysis, aqueous extraction, dessication and reconstitution, autolysis, freeze-thawing, ultrasonic disruption or the use of a french pressure cell. For a review of solubilization procedures see Grothaus (M.Sc. Thesis, The Ohio State University, 1978). The insoluble fractions were removed by centrifugation or
filtration and the remaining soluble fractions were used as antigen.

These extracts were evaluated by complement fixation (Coggeshall and Eaton, 1938; Eaton and Coggeshall, 1939; Dulaney and Morrison, 1944; Dulaney and Stratman-Thomas, 1940; Rein, et al., 1949; Prior and Kreier, 1972; Ward and Conran, 1966), fluorescent antibody procedures (Sadun and Gore, 1968; Sadun, 1969), various hemagglutination tests (Stein and Desowitz, 1964; Farshey and Kagen, 1973; Kagen, 1972) and ELISA (Voller, et al., 1975).

Similar preparations of soluble antigens were eventually fractionated using Sephadex G-200 or G-100 gel chromatography (Chavin, 1966; D'Antionio, 1966a; 1966b; Turner and McGregor, 1969; Wilson, 1969; Fife, et al., 1972; McAlister, 1972), Sephadex DEAE A-25 ion exchange chromatography (Sadun and Gore, 1968; Sadun, 1969; Chavin, 1966; Hamburger and Zuckerman, 1978), and ammonium sulfate precipitation (Davis, 1948).

These crude separation techniques yielded only 2 or 3 fractions from most plasmoidal preparations. Sephadex gel filtration, ion exchange chromatography and ammonium sulfate precipitation did seem to achieve some separation of the parasite components from hemoglobin (Wellde, et al., 1969). The various plasmoidal fractions produced by these techniques had very similar antigenic activity when they were analyzed using the serodiagnostic tests. These fractionation procedures have yielded very little useful information about the antigenic characteristics of the plasmodia.

Immunoelectrophoretic and gel diffusion techniques have been used for the identification and immunological characterization of the
antigens of various plasmodia.

Spira and Zuckerman (1962) and Zuckerman (1964) analyzed extracts of *Plasmodium vinckei* and *Plasmodium berghei* by immunoelectrophoresis and demonstrated that 6 to 8 precipitin bands developed when the antigens were reacted with antisera. Cell-free extracts of several species of plasmodia were analyzed by Zuckerman (1966) using immunodiffusion. Eight to ten precipitin arcs were observed when *Plasmodium cynomolgi* and *Plasmodium knowlesi* were diffused against homologous antisera. Banki and Bucci (1964) analyzed antigens of *Plasmodium berghei* and *Plasmodium cynomolgi* using both immunodiffusion and immunoelectrophoresis. Twelve components were identified by immunoelectrophoresis. Diggs (1964) found 5 parasite specific antigens by immunodiffusion of an extract of *Plasmodium berghei*. Sherman (1964) disrupted erythrocyte-free preparations of *Plasmodium lophurae* and then centrifuged the lysate to obtain the soluble proteins and the hemozoin. Ouchterlony and immunoelectrophoretic techniques discerned some 4 to 6 precipitating antigens present in the soluble fraction. Nine precipitating systems were observed in immunoelectrophoresis when a partially purified preparation of *Plasmodium berghei* was diffused into rabbit antisera (Corradetti, et al., 1966).

Hamburger and Zuckerman (1976) used the serum from hyperimmune rats to precipitate the antigens of *P. berghei*. This study was in conjunction with previous work classifying 12 fractions of *P. berghei* obtained by PAGE (Hamburger and Zuckerman, 1976; 1976b). Deans, et al. (1976) used crossed immunoelectrophoresis (CIE) to identify 11 major antigens of *Plasmodium knowlesi* which reacted with a pool of immune
rhesus monkey serum. Nine of these antigens were stage dependent and only two were stage independent.

Immunodiffusion, immunoelectrophoresis and more recently crossed immunoelectrophoresis, have been used to identify as many as 12 to 15 precipitating antigens. Sherman (1964) reported the identification of 8 parasite related protein fractions in a freeze-thawed preparation of Plasmodium lophurae by electrophoresis on starch gel.

Sodeman and Meuwissen (1966) fractionated the proteins in an extract of Plasmodium berghei using polyacrylamide gel electrophoresis. Free parasites were disrupted and the suspension was centrifuged for 30 minutes at 26,000 x g. A variety of different extracts were fractionated and the results showed 21 identifiable protein bands. Electrophoresis of host cell materials resulted in the formation of six bands whose migration patterns were identical to six of the bands in the experimental extract, indicating that there were a total of 16 parasite bands originating from the parasite. Chavin (1966) also studied the antigenic constituents of Plasmodium berghei by fractionation on polyacrylamide gels. He discovered at least 10 to 15 distinct protein bands using a rivanol-precipitated extract. Spira and Zuckerman (1966) compared the antigenic patterns of free parasites of simian, rodent and avian plasmodia. A range of 13-17 bands were observed for the various plasmodia. Spira and Zuckerman (1966) reported that the electrophoretic patterns of various plasmodia differed somewhat. Finerty and Dimopoullus (1968) reported the identification of 12 plasmodial components by polyacrylamide gel electrophoresis of a population of solubilized, sonically freed P. lophurae parasites. Only
4 bands were observed in hemoglobin controls. Corradetti et al. (1971) compared disc gel electrophoretic patterns of the Ististan strain with the patterns of the Vincke strain of *P. berghei*. They reported the identification of 8-11 plasmodial components but did not report the analysis of any erythrocytic controls. Kreier et al. (1976) compared polyacrylamide gel patterns of various preparations of *P. berghei* free parasites. Free parasites were released by continuous flow sonication or saponin lysis. The identification of 16-18 parasite proteins was reported.

Plasmodial preparations used in these studies probably contained at least some contamination with erythrocyte membranes (Kreier, 1977). The sensitivity of polyacrylamide gel electrophoresis is sufficient to detect even very small quantities of erythrocyte membrane proteins. Most of these studies did not include sufficient analysis of host erythrocyte membranes on the gels. Many of the studies did not even include the analysis of hemoglobin controls. At least some of the proteins identified in these studies were probably host-related.

One study in which an attempt was made to correlate immunobiological properties (Hamburger and Zuckerman, 1976a) with the immunochemical properties (Hamburger and Zuckerman, 1976b) has been reported. A soluble extract of *P. berghei* was fractionated by polyacrylamide electrophoresis and the fractions were used to immunize rats. Both protection and enhancement of infection were induced by vaccination with the various fractions.
F. THE MEROZOITE SURFACE COAT

The discovery, using electron microscopy, that merozoites have a capsule-like surface coat has caused further speculation about the importance of the immune response of the host against the merozoite. Morphological evidence for the existence of a surface coat was first derived from studies dealing with the mechanism of erythrocyte penetration. Since that time, other work has indicated that the surface coat may be directly involved in some of the important mechanisms of the immune response to malaria.

Ladda, et al. (1969) first reported that the entire surface of extracellular merozoites is coated with a granular material. While investigating the mechanism of erythrocyte penetration, using electron microscopy, they found that the adherent granular material was removed from the merozoite at the point of contact with the red cell membrane. Aggregations of surface coat material were seen on the extracellular portion of the penetrating merozoite, but little or no surface coat was observed on the intracellular portion of the parasite. These same authors postulated that this material was probably derived from the vacuole in which the parasite matured or that it was a by-product of parasite metabolism. The surface coat on merozoites incubated in fetal calf serum or in normal monkey serum was described as compact and dense, extending 20 nm from the plasma membrane (Miller, et al., 1975). Incubation in immune sera, however, caused the surface coat to thicken to 60-100 nm, to become loosely packed and partially detached. This observation has led to some controversy as to whether the surface coat is of parasite origin or whether it is simply a protein coat derived
from the surrounding serum or culture medium. Aikawa (1977; 1971) reported morphological observations of the surface coat of *Plasmodium knowlesi*. Aikawa did not observe the previously reported shedding of the coat upon erythrocyte penetration. He also suggested that the surface coat may be formed when the merozoite becomes extracellular as an effect of contact with serum immunoglobulins. This argument is based on the observation that no surface coat was found on the surface of intracellular merozoites before their release from schizonts.

Evidence for the parasitic origin of the surface coat was presented by Mason et al. (1977). They obtained merozoites *in vitro* by cultivation of infected erythrocytes and prepared them for electron microscopy without exposing the merozoites to a protein containing medium at any time. Brooks and Kreier (1978) observed surface coats on schizonts artificially released by exposure to sonic energy. They suggested that this indicated that the coat was synthesized by the parasite during intracellular growth. They reported that the surface coat on parasites which were incubated in protein free medium was indistinguishable from the surface coats of parasites prepared in serum or protein containing medium. The variations in the thickness of the surface coat in serum may be explained because the visibility of the surface coat is simply increased when the surface coat antigens react with immunoglobulins. A similar situation is observed in the "Quellung Reaction". In this reaction, treatment with type specific antibody causes pneumococcal capsular polysaccharides to become visible and swell (Davis et al., 1973). This mechanism was proposed for plasmodia by Brooks and Kreier (1978). These studies indicate that the surface coat is probably an
integral part of the parasite.

A closer examination of the surface coat showed regularly spaced T or Y shaped bristles covering the entire surface of merozoites (Bannister et al., 1977). Bannister confirmed earlier observations that a portion of the surface coat was scraped from the surface of the parasite during erythrocyte penetration. His work also supports the hypothesis of the parasitic origin of the surface coat since the surface coat was observed very early in the development of the parasite (before merozoites were exposed to external protein). The surface coat has the characteristics of an anionic glycoprotein (Bannister et al., 1977) and it is susceptible to treatment with trypsin (Miller et al., 1975). Seed et al. (1976) observed that newly formed Plasmodium simium merozoites were connected to one another as well as to the parasitophorous membrane by strands of surface material.

The presence of soluble malarial antigens in culture supernatants and in the serum of infected individuals is well documented. Antigens have been found in the sera of mice infected with Plasmodium berghei using precipitation (Seitz, 1972) and fluorescent antibody tagging (Seitz, 1976). Heat stable serum antigens (S-antigens) of Plasmodium falciparum have been found in the blood of infected Africans (Wilson et al., 1973; Wilson, 1980). The persistence of these S-antigens in the bloodstream during the course of infection (Wilson et al., 1975) and the formation of antibodies to these antigens (Wilson and MacGregor, 1973) have also been reported. It is certainly a reasonable assumption that these antigens are the same ones which were shed from the surface of merozoites during erythrocyte penetration or which were released
from rupturing schizonts. Wilson and Bartholomew (1975) disproved the theory that S-antigens leak from infected red cells and found that no S-antigens were present in the culture supernatant until the first schizonts had matured and ruptured. Collins et al. (1977) attempted to isolate S-antigens and to use them to immunize monkeys against Plasmodium knowlesi malaria. The animals immunized in this experiment showed some protection upon sporozoite challenge.

A surface coat has also been found on the sporozoite stage of malaria parasites (Cochrane et al., 1976). This surface coat was not visible when the sporozoites were incubated in a protein-free medium (TC 199). A minimal surface coat was observed on the parasites which were incubated in normal serum and a thick surface coat was observed when the parasites which were incubated in immune serum. These results were similar to those of Miller et al. (1975) who studied merozoites instead of sporozoites. In studies of sporozoites which were formalin-fixed before incubation the results indicated that the surface coat was probably made visible by the presence of immunoglobulins.

The existence of surface coats on many other microorganisms has been reported. Trypanosoma brucei contains a surface coat with antigens which undergo variation in response to antibody (Vickerman, 1978). Fluctuations in parasite numbers during the course of parasitemia and the existence of serological variants suggest the possibility of antigenic variation in the plasmodia. The surface coat would be a logical location for these variable antigens in malaria parasites.
A detailed study on the mechanical removal of surface coat antigens from *Trypanosoma congoense* has been reported (Reinwald et al., 1979). Radioactive labeling of the exterior of the parasites was utilized to determine the conditions under which the maximum amount of surface antigen could be collected. There is currently an exhaustive investigation underway to determine the properties of the surface coat of another protozoan, *Babesia bovis* (James, et al., 1981).

Surface coat which has been removed from babesia parasites has been used successfully in a number of immunizations. The antiphagocytic influence of the capsular coating on many types of bacteria, such as the pneumococci is well established. Protein A present in the walls of most *Staphylococcus aureus* has been shown to interfere with opsonization *in vitro* (Peterson, et al., 1978).

The fact that a significant portion of resistance to plasmodia is mediated by antibody has been demonstrated by passive transfer of immune serum *in vivo* (I.N. Brown, 1969; Diggs and Osler, 1969; Golenser et al., 1975; Quinn and Wyler, 1979) and *in vitro* by addition of immune serum to culture systems (Campbell et al., 1979; Reese and Motyl, 1979). There is some controversy as to the mechanisms of antibody action responsible for mediating this response. It has been suggested that schizont-infected cell agglutinins (SICA) are responsible for mediating immunity (Brown and Brown, 1965). These SICA antibodies have not been correlated with protective immunity and it is now believed that they are at least partially responsible for the induction of antigenic variation (Brown, 1971). The best correlation between functional protective immunity and a mechanism of antibody action shown
to date has been the merozoite reinvasion inhibition test. Cohen et al. (1969) measured parasite growth in vitro by measuring the incorporation of $^3$H-leucine into parasite protein. Immune serum had no effect on the development of intracellular parasites. It interrupted the cycle of growth by inhibiting the reinvasion of erythrocytes by merozoites only after complete development of the schizonts. This merozoite reinvasion inhibition assay has been positively correlated with immunity to parasite challenge in monkeys that had undergone drug-controlled infections (Cohen, Butcher, and Mitchell, 1972; Butcher and Cohen, 1972) or merozoite vaccination (Butcher et al., 1977). In culture, immune serum from Gambians also inhibited erythrocyte reinvasion but did not affect the intracellular growth of parasites as measured by incorporation of $^3$H-leucine (Wilson and Phillips, 1976). Other work has shown that antibody may also be responsible for enhanced phagocytosis of free parasites (Chow and Kreier, 1972; Brooks and Kreier, 1978), by cytophilic interaction with macrophages (Green and Kreier, 1978) or opsonic coating of free parasites (Hamburger and Kreier, 1975). These various mechanisms are certainly not exclusive of one another and in fact probably all interact to contribute to elimination of the parasites.

There has recently been information from a variety of sources indicating that merozoite surface antigens may be important in the implementation of these responses. The first evidence that a portion of the immune response of the host might be directed against the surface coat was presented by Miller et al. (1975). They found that immune serum agglutinated Plasmodium knowlesi merozoites in culture by
binding the surface coats of adjacent parasites. Clumps of merozoites were able to attach to erythrocytes but generally did not penetrate them. Free parasites were able to penetrate cells even in the presence of immune serum. Another possible explanation is that antibody acts by preventing the dispersal of merozoites at the time of schizont disruption (Green, et al., 1981). The observation by Green et al. that schizonts fail to disperse in the presence of immune serum would explain how immune serum could inhibit merozoite reinvasion of erythrocytes. Both schizont inhibition and merozoite agglutination indicate strongly that the specific mechanism of antibody action is a cross-linking of the surface coat antigens found on mature merozoites.

The surface coat (capsule) on Plasmodium berghei merozoites appears to be antiphagocytic (Brooks and Kreier, 1978). Electron microscopy showed that the surface coat was present on developing merozoites in the host cell but was not present on trophozoites freed from host erythrocytes by sonication. Trophozoites, but not merozoites, were readily phagocytized by peritoneal macrophages in normal serum. Incubation in immune serum apparently opsonized merozoites and the antiphagocytic effect of the capsule was overcome. This work has presented a more detailed explanation of the mechanism of phagocytosis of malaria parasites than did previous work.

Merozoite vaccinations have shown a higher degree of protection than any other vaccinations to date. The merozoite surface coat may be responsible for the induction of this immunity.

There is evidence that the surface coat interacts with immune serum to initiate agglutination of free merozoites and inhibition of
merozoite dispersal at the time of their release from schizonts. The surface coat appears to be antiphagocytic unless it is coated with immune serum.

Soluble material present in culture supernatants and in the serum of infected hosts may also be derived from the merozoite surface coat. The importance of surface coat or capsular antigens in the host-parasite interactions of many other microorganisms has been well documented.
MATERIALS AND METHODS

Parasites. The Plasmodium berghei (WR) strain used in this study was obtained from M. Aikawa (Case Western Reserve University, Cleveland, Ohio). This strain, which originated from the Walter Reed Army Institute of Research, is highly pathogenic in mice and causes death, usually within one week of the onset of patency. Weanling rats may die when infected with this strain, but adult rats usually survive.

A pool of parasitized mouse erythrocytes in Alsever's solution plus 10% (v/v) glycerol was stored in liquid nitrogen (-193 C). This method of maintenance was chosen instead of serial passage in mice to reduce the possibility of antigenic drift in the parasite reference strain.

The Plasmodium falciparum (Honduran strain) used in this study was obtained from Dr. Fred Hink (Department of Entomology, The Ohio State University, Columbus, Ohio).

Animals. Outbred swiss albino mice were used to recover parasites from liquid nitrogen storage. The parasites were injected intraperitoneally after the inoculum had been thawed in tepid water. Similar mice were used for testing the infectivity of the cultured P. berghei.

Adult Sprague Dawley rats (Fisher 344, Charles River Breeding Laboratories, Wilmington, Mass.) were used as the source of P. berghei free parasites and of the normal and infected erythrocytes used for measuring the uptake of radiolabelled metabolites and analyzed by
polyacrylamide gel electrophoresis (PAGE). Inbred male CDF rats were used in all vaccination experiments, for antigen production, as the source of challenge inocula, and as the test animals. The vaccination experiments were carried out in young adult male rats weighing between 70 and 90 grams. The animals were allotted to the various groups randomly.

New Zealand White rabbits were used for the production of antiserum for the immune precipitation experiments.

**Harvesting of Free Parasites.** Blood from infected rats (approximately 30% to 50% parasitemia) was drawn by cardiac puncture into cold Alsever's solution. Parasitemia was calculated by preparing a blood smear from the tail of the animal, staining the smear with giemsa stain, and counting the number of infected erythrocytes per 100 erythrocytes. To attain this high parasitemia, the reticulocyte count was increased by injection of phenylhydrazine hydrochloride (1.5% aqueous solution). A dose of 30 mg/kg of body weight was given on days 0 and 2 of the harvest, as described by Kreier, et al. (197b).

The erythrocytes from the infected rats were washed twice in Alsever's solution by centrifugation at 650 x g for 10 minutes and diluted to yield a 10% suspension (v/v) of erythrocytes. This suspension was passed through a column of Whatman no. 1 powdered filter paper packed lightly in a 50 ml syringe to remove leucocytes. The erythrocytes were disrupted by treatment in a continuous-flow sonication system (29.6 ml/min; 20kHz) by methods previously described (Prior and Kreier, 1972a and 1972b).
The suspension was then spun at 650 x g for 10 minutes to remove unbroken erythrocytes, fragments of erythrocytes and large free parasites. The supernatant, containing predominantly small free parasites, was collected and centrifuged at 6000 x g for 10 minutes, yielding a brown pellet of parasites. For some experiments, the pellet was resuspended in physiological saline (0.15M NaCl) and then washed once by centrifugation for 10 minutes at 6,000 x g (Hamburger and Kreier, 1975; Kreier, et al., 1976).

Freeze-thawing. Suspensions of free parasites in physiological saline or Alsever's solution were distributed in 8 x 75 mm test tubes and were immersed in a dry ice-acetone bath until the parasite suspension was frozen solid. The test tubes containing the frozen parasites were then swirled in a 37 C water bath until there were no ice crystals visible in the suspension. This procedure was repeated for each cycle of freeze-thawing. Three times freeze-thawed free parasites or infected erythrocytes were separated into supernatant (soluble fraction) and pellet (membranous fraction) by centrifugation for 30 minutes at 30,000 x g.

Harvesting of Membranes. Uninfected erythrocytes or P. berghei infected erythrocytes were washed twice in Alsever's solution and the leucocytes were removed as previously described. The cells were lysed by addition of ice cold double distilled water (DDW) which had been bubbled for 30 minutes with CO₂. To each volume of packed erythrocytes 10 volumes of CO₂ rich DDW were added.
The membranes were collected by centrifugation at 10,000 x g for 15 minutes and washed three times with the CO₂ saturated water or until the supernatant was relatively free of hemoglobin. No attempt was made to separate parasite membranes from erythrocyte membranes in infected cell preparations.

**Washoff Antigen.** The washoff antigen was prepared by suspending about 4 x 10⁹ free parasites in 2 ml of cold saline. The suspension was spun gently with a magnetic stir bar and plate in the cold for 2 hours. The washed free parasites were then separated from the released material by centrifuging at 30,000 x g for 30 minutes. Hemocytometer counts of parasites before and after washing showed no significant change in the number of intact parasites. In the immunization experiments, the dose of washoff antigen was that amount of material contained in 0.25 ml of wash water.

**Protein Determinations.** The amount of protein contained in the vaccine preparations was determined according to the procedure of Lowry et al. (1951). The Bio-Rad Protein Assay was used to determine the protein content of samples which were analyzed by PAGE.

**Vaccinations.** All vaccinations were done in 70 to 90 gram male CDF rats. Vaccine doses were 5 x 10⁸ free parasites or the soluble or insoluble fraction of 5 x 10⁸ free parasites or the material washed from the surface of 5 x 10⁸ free parasites. Uninfected rat erythrocyte membranes were used as a control vaccine. Vaccination was by
intramuscular injection of the antigen with saponin (0.3125 mg/dose) as adjuvant. Each vaccination group (4 rats/group) was given one dose of vaccine. The rats were challenged 2 weeks later by intravenous injection of $10^4$ parasitized erythrocytes. Blood smears were prepared daily and stained with Giemsa stain. Infection was monitored by counting the number of parasitized cells per 10,000 erythrocytes. A detailed description of the vaccination procedure has been published elsewhere (Saul and Kreier, 1978; Grothaus and Kreier, 1980).

Preparation and Culture of Erythrocytes. *P. berghei* infected and normal rat erythrocytes were obtained from Sprague Dawley rats. Blood from infected, phenyl hydrazine (ph)-treated rats (as previously discussed) was harvested when the parasitemia reached approximately 20%. Uninfected, phenyl hydrazine (ph) treated rats (control) had about 50% reticulocytes when the cells were harvested. Blood from *P. berghei* infected rats (parasitemia 1-2%) and uninfected rats (1-2% reticulocytes) which did not receive phenyl hydrazine were also used in some uptake experiments.

Blood was drawn into heparin (8 units/ml final volume) under sterile conditions by cardiac puncture. The heparin was used to prevent clotting. The blood was diluted 1:6 in Temin's Modified Minimal Essential Medium (tMEM) which had been supplemented with 10% heat inactivated fetal calf serum, except in the methionine uptake experiments when 1/10 the normal methionine supplement was added. White blood cells were removed by filtration through the packed filter paper column. The column was washed with phosphate buffered saline
(PBS) before and after the addition of the blood cells.

This procedure yielded a preparation with a final concentration of about $1 \times 10^9$ cells/ml.

The $P. falciparum$ infected RBC's were maintained by serial passage in culture. $P. falciparum$ infected erythrocytes (parasitemia 3%) or normal human RBC's were washed and resuspended in Temin's Modified Minimal Essential Medium (tMEM) or RPMI 1640 at the same final concentration as were the $P. berghei$ infected erythrocytes.

Two mls of the appropriate cell suspension was placed in a 4.0 ml petri dish and incubated at 37 C in a 5% CO$_2$ atmosphere.

**Exposure to Radiolabelled Metabolites.** L-$(^{35}S)$-methionine (1206 Ci/m mole), (6-$^3$H)-thymidine (5-15 Ci/m mole) and (6-$^3$H)-uridine (20-30 Ci/m mole) were obtained from New England Nuclear (Boston, Mass.). D-$(^3$H)-glucosamine hydrochloride (22 Ci/m mole) was obtained from Amersham Corporation (Arlington Heights, Ill.).

The volume of each radiolabelled metabolite which was added to the cultures was adjusted so that the final concentration was always 20 uCi/ml of cell suspension.

In most of the uptake experiments, radiolabelled metabolites were added to the dishes at time 0 and small increments were removed and analyzed at various intervals (continuous exposure to label). Another method involved initiating 4 identical cultures (for example, A-D). At time 0, label was added to culture A. After a designated period of incubation, culture A was terminated and harvested as described below, while label was simultaneously added to culture B and so on (pulse label).
Sampling. The cells were gently resuspended and the entire contents of the culture dishes or 0.4 ml samples from the dishes were removed and centrifuged for 3 minutes at 10,000 x g (Beckman Microfuge). The culture supernatants were removed at this time and saved for counting or analysis on polyacrylamide gels. The pellets were washed 2 times in 1 ml of phosphate buffered saline (100 ug/ml PMSF) and resuspended in 200 ul of the SDS sample buffer (0.0625 M Tris-HCl (pH 6.8), 3% SDS, 20% glycerol and 5% 2-mercaptoethanol).

Giemsa smears. Blood smears were prepared and stained with Giemsa stain each time a sample was taken from a culture dish. Relative parasitemias and the condition of the blood cells were observed.

Infectivity of Cultured P. berghei Infected Erythrocytes. Four mice were injected with 0.2 ml of blood from an infected culture (20% initial parasitemia) at 0, 10, 20, 48 and 72 hours after initiation of the culture. Blood smears were prepared and the parasitemia of the mice was monitored for 10 days after they received the injections.

Liquid Scintillation Counting. A 5 ul sample of each solubilized pellet or supernatant was added to 1 ml of DDW and 100% trichloroacetic acid (TCA) was added to a final concentration of 25% TCA. The samples were then left on ice for 30 minutes to allow for precipitation.

The TCA insoluble material was separated from the free isotope by filtration on Reeve Angel glass fiber filter paper (Whatman, Inc., Clifton, N.J.). The filters were washed with 5% TCA followed by 95%
ethanol.

After drying, the filters were immersed in 8.0 ml scintillation cocktail (Liquiflour Scintillation Fluid, New England Nuclear) and counted using a Beckman Liquid Scintillation System (Model LS-355).

**Preparation of Radiolabelled Antigens.** The antigens used in the immune precipitation experiments were prepared by exposing *P. berghei* infected rat erythrocytes (20% parasitemia) or normal rat erythrocytes to L-(35S)-methionine using the culture conditions which have been previously described.

After 10 hours in culture, the erythrocytes were harvested (10,000 x g for 3 minutes) and washed 2 times in PBS containing phenylmethyl-sulfonylfluoride (PMSF) (Sigma Chemical Corp., St. Louis, Mo.) (100 ug/ml) as a protease inhibitor. The cell pellets were then resuspended in a small amount of Radioimmunoprecipitation buffer (RIPA) (150 mM NaCl, 10 mM Tris-HCl pH 7.2, 1% sodium deoxycholate, 1% Triton x-100, 0.1% SDS, 100 ug/ml PMSF). The samples were placed in an ice bath for 10 minutes, sonicated for 1 minute and vortexed violently. The extracts were used immediately or stored at -70 C. Before use, the extracts were centrifuged at 10,000 g for 2 minutes to remove any particulate material.

**Preparation of Antiserum.** Rabbits were injected with various preparations of *P. berghei* infected and normal rat erythrocytes, *P. berghei* free parasites, and washoff antigen. The rabbits were given a series of intramuscular and intradermal injections at approximately 2
week intervals. The initial preparations were mixed with Freund's complete adjuvant to increase the immune response of the rabbits.

Serum from rats which had been infected with P. berghei was also analyzed. The serum was collected from rats at various times during the course of infection and from rats which had recovered from infection with P. berghei.

Precipitation of Immune Complexes Using Staphylococcus Aureus Protein A. This procedure was modified from those of Bodemer et al., (1980) and Suh et al., (1980). A volume of 15 ul of L-(35S)-methionine labelled antigen was reacted with 10 ul undiluted antiserum in the presence of 15 ul of 2% D-L methionine. The mixture was incubated for 1 hour at room temperature to allow for the formation of antigen-antibody complexes.

The reaction volume was increased by the addition of 200 ul RIPA buffer before adding 15 ul (10% v/v) formalized, Protein A bearing S. aureus immunoabsorbant (The Enzyme Center, Boston, Mass.) (Kessler, 1975). The bacteria and immune complexes were allowed to react for 5 minutes and removed by centrifugation at 10,000 g for 2 minutes. The pellet was washed 2 times in RIPA buffer and finally resuspended in 100 ul SDS sample buffer. After the samples were placed in boiling water for 5 minutes to dissociate the immune complexes, the bacteria were removed by centrifugation at 10,000 x g for 2 minutes. The supernatants were analyzed by PAGE.
**Polyacrylamide Gel Electrophoresis.** The PAGE system used in this study was adopted from that of Laemmli (1973). Acrylamide, bis-acrylamide, ammonium persulfate, temed, SDS, and bromphenol blue were obtained from Bio-Rad Laboratories (Richmond, Calif.).

Stacking gels were 4% acrylamide. Separation gels were 7.5%, 10%, 12% or 10-20% gradients depending on the particular experiment.

Constant voltage of 75 volts/gel (Bio Rad Power Supply Model 500/200) was applied until the tracking dye (bromphenol blue) had migrated to the bottom of the gel.

**Molecular Weight Determinations.** Molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, CA). The relative mobilities of the standards were determined using our PAGE system. The average values of at least 10 trials were plotted versus the molecular weights to construct the standard curves shown in Appendix II (Figure 27).

**Staining of Polyacrylamide Gels.** Proteins were stained with Coomassie Brilliant Blue R-250 (Garvey, 1977) or silver-based color stain (Sammons et al., 1981).

**Gel Processing and Autoradiography.** Gels were placed in 10% glacial acetic acid for at least 30 minutes in order to fix the proteins. The gels were then agitated gently in 2 changes of dimethylsulfoxide (DMSO) (Curtis Matheson Scientific Inc., Cleveland, Ohio) for 30 minutes each followed by 3 hours in a solution of DMSO
containing 20% 2,5 diphenyloxazole scintillator (Mallinckrodt Inc., Paris, Kentucky). The gels were washed 2 times in DDW and dried (2-3 hours) on a Bio-Rad Model 224 Slab Gel Dryer.

The dried gels were placed on Kodak X-ray film (Kodak X-OMAT XAR-5) and exposed from 12 hours to 3 weeks at -70 C. The films were developed and fixed with Kodak reagents.
RESULTS

A. Polyacrylamide Gel Electrophoresis of The Components of Plasmodium berghei Free Parasites, Infected Erythrocytes and Uninfected Erythrocytes; Coomassie Brilliant Blue Stain and Silver Based Color Stain.

The P. berghei free parasites used in this analysis were prepared by our standard continuous flow sonic technique (Kreier et al., 1975). P. berghei free parasites were compared to infected erythrocytes and uninfected erythrocytes by electrophoresis in 10% polyacrylamide gels. The gel shown in Figure 1 was stained with Coomassie blue R-250. Erythrocyte membrane contamination in the free parasite preparation was indicated by the presence of spectrin. Spectrin from the erythrocyte membrane appears as the fairly distinct double band at the top of the gel. The molecular weights of these membrane proteins are in the 225,000 to 250,000 dalton range (Fairbanks et al., 1971; Marchesi and Furthmayr, 1976). Most of the hemoglobin contained in the sample of soluble material from uninfected erythrocytes (Figure 1, tracks 1 and 2) was apparently broken down into a 32,000 dalton subunit although there is also a band which probably represents hemoglobin having a molecular weight of 64,000 daltons. Both infected and uninfected erythrocyte membrane preparations (Fig. 1, tracks 9 and 10) contained some components that were absent from the free plasmodia or present in amounts undetectable by this procedure. These components had molecular weights of approximately 140,000, 77,000 and 64,000 daltons. The plasmodial preparations (track 11, free parasites, track 10, infected
Figure 1. Coomassie blue stain of a 10% polyacrylamide gel showing patterns produced by electrophoresis of preparations of *P. berghei* free parasites, infected erythrocytes, uninfected erythrocytes and hemoglobin. Tracks 1 and 2, hemoglobin; tracks 3, 6 and 9, uninfected erythrocytes; tracks 4, 7 and 10, infected erythrocytes; tracks 5, 8 and 11, free parasites; tracks 12 and 13, molecular weight markers.
erythrocytes) had one unique component which was detectable by staining with Coomassie blue. It had a molecular weight of approximately 52,000 daltons.

The gel shown in Figure 2 also shows the patterns produced by electrophoresis of P. berghei free parasites, P. beghei infected erythrocytes and uninfected erythrocytes after electrophoresis on a 10% polyacrylamide gel. This gel was stained using the silver based color stain (Sammons et al., 1981). The samples which were analyzed on this gel were identical to the samples analyzed on the gel shown in Figure 1. There were at least 3 bands unique to the infected preparations (track 2, free parasites; track 3, infected erythrocytes) on the gel shown in Figure 2. They had molecular weights of approximately 115,000, 57,000 and 28,000 daltons. There was also a set of 3 or 4 very faint bands which appeared only in the free parasite preparation (track 2). Their molecular weights were in the 65,000 to 85,000 dalton range. The large number of bands which appear in the hemoglobin samples (tracks 5 and 6) probably represent trace amounts of contamination from other components of the erythrocyte.

B. Dynamics of the Uptake of Isotopically Labelled Metabolites
(3H-thymidine, 3H-glucosamine and 35S-methionine) by Plasmodium berghei Infected and Uninfected Erythrocytes in Culture.

3H-uridine and 3H-thymidine. Figure 3 shows the number of counts incorporated by P. berghei infected erythrocytes and uninfected erythrocytes after they were cultured in the presence of 3H-uridine or
Figure 2. Silver based color stain of a 10% polyacrylamide gel showing patterns produced by electrophoresis of preparations of *P. berghei* free parasites, infected erythrocytes, uninfected erythrocytes, and hemoglobin. Tracks 1 and 7; molecular weight markers; tracks 2 and 8, free parasites; track 3, infected erythrocytes; track 4, uninfected erythrocytes; tracks 5 and 6, hemoglobin.
Figure 3. Dynamics of $^3$H-uridine and $^3$H-thymidine uptake by *P. berghei* infected (IRBC) and uninfected erythrocytes (NRBC) (continuous exposure to label). Counts represent the activity contained in 5 ul samples of washed erythrocytes.
FIGURE 3

[Line graph showing CPM over hours for different conditions.

- IRBC; ^3H-URIDINE
- IRBC; ^3H-THYMIDINE
- NRBC; ^3H-URIDINE
- NRBC; ^3H-THYMIDINE]
The amount of $^3$H-thymidine incorporated by *P. berghei* infected erythrocytes was approximately 2 times the amount incorporated by the uninfected erythrocytes. The amount of $^3$H-thymidine incorporated by *P. berghei* infected erythrocytes was approximately equal to the amount incorporated by the uninfected erythrocytes. Most incorporation took place during the first 5 hours in culture. There was very little additional incorporation of either isotope after the first 5 hours in culture (Table 1; Figure 3).

$^3$H-glucosamine. Figure 4 shows the number of counts incorporated by *P. berghei* infected erythrocytes and uninfected erythrocytes after they were cultured in the presence of $^3$H-glucosamine. The amount of $^3$H-glucosamine incorporated by *P. berghei* infected erythrocytes was approximately 5 times the amount incorporated by uninfected erythrocytes after 5 hours exposure.

The counts contained in the material which was TCA precipitated from 5 ul samples of the culture supernatants did not differ significantly between cultures containing infected or uninfected erythrocytes (Table 2; Figure 4).

Figure 5 shows an autoradiograph of a 10% polyacrylamide gel containing the samples prepared from *P. berghei* infected and uninfected erythrocytes after they were cultured for 5 hours in the presence of $^3$H-glucosamine. There were no $^3$H-glucosamine labelled components visible on the tracks containing samples prepared from uninfected erythrocytes (tracks 3 and 4). There were two $^3$H-glucosamine labelled components resolved on the tracks containing the samples prepared from infected erythrocytes (tracks 1 and 2). They had molecular weights of
Figure 4. Dynamics of $^3$H-glucosamine uptake by *P. berghei* infected (IRBC) and uninfected erythrocytes (NRBC) (continuous exposure to label). Counts represent the activity contained in 5 ul samples of washed erythrocytes (P) or TCA precipitated supernatants (SN).
FIGURE 4

CPM
200
400
600
100

1 2 3 4 5
HOURS

IRBC (P)
IRBC (SM)
MRBC (P)
MRBC (SM)
Figure 5. Autoradiograph of a 10% polyacrylamide gel containing *P. berghei* infected and uninfected erythrocytes after 5 hours exposure to $^3$H-glucosamine. Infected erythrocytes, tracks 1 and 2; uninfected erythrocytes, tracks 3 and 4.
FIGURE 5

<table>
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<488
<45
<31
<21
approximately 50,000 and 200,000. In addition, there was some labelled material which did not enter the resolving gel on the tracks containing the samples prepared from infected erythrocytes.

$^{35}$S-methionine. Figure 6 shows the number of counts incorporated by $P$. berghei infected and uninfected erythrocytes after they were cultured in the presence of $^{35}$S-methionine. The amount of $^{35}$S-methionine incorporated by $P$. berghei infected erythrocytes was 2-3 times as great as the amount incorporated by uninfected erythrocytes. The amount of incorporation of $^{35}$S-methionine was roughly proportional to the parasitemia in infected preparations. In uninfected cultures, there was more incorporation of $^{35}$S-methionine by reticulocytes than by mature erythrocytes. TCA precipitated polypeptides in supernatants from cultures containing infected erythrocytes appeared to have incorporated more $^{35}$S-methionine than the TCA precipitated polypeptides in supernatants from cultures containing uninfected erythrocytes (Table 3; Figure 6).

Figure 7 shows an autoradiograph of a 10% polyacrylamide gel containing samples of $P$. berghei infected and uninfected erythrocytes which were taken at various times after continuous exposure to $^{35}$S-methionine in culture. The uninfected erythrocytes incorporated $^{35}$S-methionine into only a few components which were revealed on the gel (tracks 8-14). A moderately large amount of low molecular weight material was also labelled. This material moved with the tracking dye. The infected erythrocytes incorporated the $^{35}$S-methionine into a large number of components which were resolved on the gel (tracks 1-7).
Figure 6. Dynamics of $^{35}$S-methionine (NRBC) uptake by *P. berghei* infected (IRBC) and uninfected erythrocytes (continuous exposure to label). $+\text{PH}$ = phenyl hydrazine treated to increase reticulocytosis. $-\text{PH}$ = no phenyl hydrazine treatment. Counts represent the activity contained in 5 ul samples of washed erythrocytes (P) or TCA precipitated supernatants (SN).
Figure 7. Autoradiograph of a 10% polyacrylamide gel showing samples of P. berghei infected or uninfected erythrocytes taken at various times after continuous exposure to $^{35}$S-methionine in culture. Tracks 1-7 infected erythrocytes at times 0.25, 5, 10, 15, 20, 48 and 72 hours; tracks 8-14 uninfected erythrocytes at times 0.25, 5, 10, 15, 20, 48 and 72 hours.
Figure 8 illustrates the results of the $^{35}\text{S}$-methionine uptake experiments in which the erythrocytes were exposed to the label in 5 hour pulses. The number of counts incorporated by the erythrocytes (Table 4; Figure 8) and an autoradiograph of a polyacrylamide gel containing samples of the pulse labelled erythrocytes (Figure 9) indicate that nearly all of the $^{35}\text{S}$-methionine incorporation took place during the first 5 hours in culture, slight incorporation took place during the second 5 hour period and very little incorporation took place during the third and fourth 5 hour periods. This was true in both infected and uninfected erythrocytes.

The two exposures of the autoradiograph (Fig. 9a, 48 hours; Fig. 9b, 5 days) are presented to show that although a much smaller amount of label was incorporated by infected erythrocytes during the second 5 hour period (Fig. 9b, track 2), the labelled components appear to be identical to those which were labelled during the first 5 hour period (Fig. 9a, track 1).

C. Comparison By Electrophoresis in Polyacrylamide Gel of $^{35}\text{S}$-methionine Labelled Components to Coomassie Brilliant Blue Stained Components of *Plasmodium berghei* Infected and Uninfected Erythrocytes

Figures 10, 11 and 12 show autoradiographs and Coomassie blue stains of polyacrylamide gels containing samples of *P. berghei* infected erythrocytes and uninfected erythrocytes after they had been cultured in the presence of $^{35}\text{S}$-methionine. The gel shown in Figure 10 is 7.5% acrylamide. The gels shown in Figures 11 and 12 are 10-20% acrylamide.
Figure 8. Dynamics of the uptake of $^3$S-methionine by P. berghei infected (I) and uninfected erythrocytes (U) by exposure in 5 hour pulses. The counts represent the activity contained in 5 ul samples of washed erythrocytes.
Figure 9. Autoradiograph of a 10% polyacrylamide gel containing *P. berghei* infected and uninfected erythrocytes after exposure to $^{35}$S-methionine in 5 hour pulses. Tracks 1-4, infected erythrocytes, track 1, 0-5 h; track 2, 5-10 h; track 3, 10-15 h; track 4, 15-20h. Tracks 5-8 uninfected erythrocytes, track 5, 0-5 h; track 6, 5-10 h; track 7 10-15 h; track 8, 15-20 h. Track 9, molecular weight markers. Autoradiograph shown in A was exposed for 48 hours, B was exposed for 5 days.
Figure 10. Autoradiographs and Coomassie blue (CB) stains of the components of *P. berghei* infected and uninfected erythrocytes after exposure to $^{35}$S-methionine and electrophoresis on a 7.5% polyacrylamide gel. Track 1, 6, 15, 20, molecular weight markers; tracks 2-5, autoradiograph of infected erythrocytes; tracks 7-10, CB stain infected erythrocytes; tracks 12-15, autoradiographs of uninfected erythrocytes; tracks 16-20, CB stain of uninfected erythrocytes.
FIGURE 10
Figure 11. Autoradiography and Coomassie blue (CB) stains of the components of P. berghei infected and uninfected erythrocytes after exposure to $^{35}$S-methionine and electrophoresis on a 10-20% polyacrylamide gradient. Tracks 5 and 19, molecular weight markers; tracks 1, 2, 3, 4, autoradiographs of infected erythrocytes; tracks 6, 7, 8, 9, CB stains of infected erythrocytes; tracks 10, 11, 12, 13, autoradiographs of uninfected erythrocytes; tracks 15, 16, 17, 18, CB stain of uninfected erythrocytes.
Figure 12. Autoradiographs and Coomassie blue (CB) stains of the components of *P. berghei* infected and uninfected erythrocytes after exposure to $^{35}$S-methionine and electrophoresis on a 10-20% polyacrylamide gradient. Tracks 1, 2, 3, 7, 8, autoradiographs of infected erythrocytes; tracks 4, 5, 6, autoradiographs of uninfected erythrocytes; tracks 9, 10, 11, CB stains of infected erythrocytes; tracks 12-16, CB stains of uninfected erythrocytes; track 17, histones; track 18, molecular weight markers.
The Coomassie blue stains of the infected erythrocytes (Fig. 10, tracks 7, 8, 9, 10; Fig. 11, tracks 6, 7, 8, 9; Fig. 12, tracks 12-16) were very similar in appearance to the Coomassie blue stains of the uninfected erythrocytes (Fig. 10, tracks 16, 17, 18, 19; Fig. 11, tracks 15, 16, 17, 18; Fig. 12, tracks 9, 10, 11). Most of the components detectable by staining with Coomassie blue were probably host-related. Large molecular weight components of the erythrocyte membrane (probably spectrin) are seen as two distinct bands at the top of the tracks containing either infected or uninfected erythrocytes. The heavy band near the 66,000 molecular weight marker is hemoglobin (64,000 daltons). Hemoglobin subunit dimers (32,000) also appear as very dark bands on the two gradient gels (Figs. 11 and 12). The resolution on the gel shown in Figure 11 is sufficient that it is possible to distinguish between the $A_2$ and $B_2$ subunits of hemoglobin.

A comparison of the autoradiographs of the $^{35}$S-methionine labelled infected erythrocytes (Fig. 10, tracks 2, 3, 4, 5; Fig. 11, tracks 1, 2, 3, 4; Fig. 12, tracks 1, 2, 3, 7, 8) to the autoradiographs of $^{35}$S-methionine labelled uninfected erythrocytes (Fig. 10, tracks 11, 12, 13, 14; Fig. 11, tracks 10, 12, 13, 14; Fig. 12, tracks 4, 5, 6) shows that it is possible to identify a number of parasite related components by labelling with $^{35}$S-methionine. The only $^{35}$S-methionine labelled components which appeared on the autoradiographs of the uninfected erythrocytes were hemoglobin (molecular weight 64,000) and a relatively dense band of small molecular weight proteins or breakdown products (molecular weight less than 15,000) which migrated with the dye front.
Autoradiographs of the tracks containing the *P. berghei* infected erythrocytes showed between 20 and 30 $^{35}$S-methionine labelled components with molecular weights ranging from approximately 20,000 to 300,000 daltons.

Figure 13 shows Coomassie blue stains and autoradiographs of polyacrylamide gels containing TCA precipitated samples of the supernatants from $^{35}$S-methionine labelled cultures. The Coomassie blue stained supernatants from *P. berghei* infected cultures (tracks 3 and 4) and uninfected erythrocyte cultures (tracks 7 and 8) both had a very dense hemoglobin band and an identical set of 5 smaller molecular weight components. Autoradiographs of the supernatants from infected cultures (tracks 1 and 2) and supernatants from uninfected cultures (tracks 5 and 6) showed only some labelled components with molecular weights of less than 30,000 and labelled hemoglobin. There were no components appearing on these gels which were unique to the supernatants from infected erythrocyte cultures.

D. A Comparison of the $^{35}$S-methionine Labelled Components Contained in Four Different Preparations of *P. berghei* Infected Erythrocytes and Determination of Molecular Weights.

Figure 14 shows an autoradiograph of a polyacrylamide gel containing four preparations of $^{35}$S-methionine labelled *P. berghei* infected erythrocytes. Preparation A (tracks 1, 2, 3), Preparation B (tracks 4, 5, 6), Preparation C (tracks 7, 8, 9) and Preparation D (tracks 10, 11, 12) were each labelled during the course of a different
Figure 13. Autoradiographs and Coomassie blue (CB) stains of supernatants (SN) from infected and uninfected erythrocyte cultures. The gel is 7.5% polyacrylamide. Tracks 1 and 2, autoradiograph of infected culture SN; tracks 3 and 4, CB stain of infected SN; track 5 and 6, autoradiograph of uninfected culture SN; tracks 7 and 8, CB stain of uninfected SN; track 9, molecular weight markers.
Figure 14. Autoradiograph of a 10% polyacrylamide gel containing samples of four different preparations of *P. berghei* infected erythrocytes after 10 hours exposure to $^{35}$S-methionine. Tracks 1, 2, 3, preparation A; tracks 4, 5, 6, preparation B; tracks 7, 8, 9, preparation C; tracks 10, 11, 12, preparation D.
$^{35}$S-methionine uptake experiment. Each preparation of *P. berghei* infected erythrocytes was harvested from a different animal with a parasitemia of approximately 20%. Although there are differences in the total radioactivity contained in the various samples, the number and relative intensity of the $^{35}$S-methionine labelled components is nearly identical in the four different preparations.

Figure 15 shows an autoradiograph of a 10% polyacrylamide gel illustrating the labelled components of *P. berghei* infected erythrocytes after 10 hours exposure to S-methionine. There are 32 components which were detectable by this procedure ranging in molecular weight from 18,000 to 300,000 daltons. The standard curve used in the molecular weight determinations can be found in Appendix II.

E. Dynamics of $^{35}$S-methionine Uptake by *P. falciparum* Infected Erythrocytes or Uninfected Erythrocytes in Temin's Modified Minimal Essential Media or RPMI 1640; Comparison of $^{35}$S-methionine labelled Components of *P. falciparum* to $^{35}$S-methionine labelled Components of *P. berghei*.

Figure 16 shows the number of counts incorporated by *P. falciparum* infected erythrocytes and normal human erythrocytes after they were exposed to $^{35}$S-methionine for 12 hour periods. The erythrocytes were cultured in either Temin's Modified Minimal Essential Media (MEM) or RPMI 1640 in petri dishes by the Trager and Jenson method (Trager and Jenson, 1980). The amount of $^{35}$S-methionine incorporated by infected erythrocytes was greater than the amount of $^{35}$S-methionine incorporated
Figure 15. Autoradiograph of a 10% polyacrylamide gel showing the molecular weights of the $^{35}$S-methionine labelled components of *P. berghei* infected erythrocytes. See Appendix II for standard curve used for determination of the molecular weight.
Figure 16. Dynamics of the uptake of $^{35}$S-methionine by *P. falciparum* infected erythrocytes (IRBC) and normal human erythrocytes (NRBC) suspended in cultures containing Temin's Modified Minimal Essential Media (MEM) with a reduced amount of cold methionine or RPMI 1640 (12 hr pulse labels). Counts represent the activity contained in 5 ul samples of washed erythrocytes.
by uninfected erythrocytes in both MEM and RPMI 1640. The amount of 
\[ ^{35}S \text{-methionine} \]
incorporated by \textit{P. falciparum} infected erythrocytes grown in MEM was approximately equal to the amount of \[ ^{35}S \text{-methionine} \]
incorporated by \textit{P. falciparum} infected erythrocytes grown in RPMI 1640 (Table 5, Figure 6).

Figure 17 shows an autoradiograph of a 10% polyacrylamide gel on which samples of \textit{P. falciparum} infected and uninfected human erythrocytes were analyzed. These erythrocytes were cultured in the presence of \[ ^{35}S \text{-methionine} \] for 12 hours in RPMI 1640. The \textit{P. falciparum} infected erythrocytes (tracks 1 and 2) contained a large number of \[ ^{35}S \text{-methionine} \] labelled components. The uninfected human erythrocytes (tracks 3 and 4) did not have any \[ ^{35}S \text{-methionine} \] labelled components detectable by this procedure.

Figure 18 shows an autoradiograph of a 10% acrylamide gel in which the \[ ^{35}S \text{-methionine} \] labelled components of \textit{P. falciparum} infected erythrocytes were compared to the \[ ^{35}S \text{-methionine} \] labelled components of \textit{P. berghei} infected erythrocytes. Many similarities and at least one difference between the two organisms were observed. Most major components of the two plasmodia had identical molecular weights. At least one major component of \textit{P. falciparum} infected cells (tracks 1, 2, 3) was not apparent in the \textit{P. berghei} infected cells (tracks 4, 5, 6). It had a molecular weight of approximately 160,000 daltons.
Figure 17. Autoradiographs of a 10% polyacrylamide gel containing samples of *P. falciparum* infected erythrocytes and normal human erythrocytes after exposure to $^{35}$S-methionine. The erythrocytes were cultured for 12 hours in RPMI 1640. Tracks 1 and 2; *P. falciparum* infected erythrocytes; tracks 3 and 4, normal human erythrocytes.
Figure 18. Autoradiograph of a 10% polyacrylamide gel comparing the 35S-methionine labelled components of *P. falciparum* infected erythrocytes (tracks 1, 2, 3) to the 35S-methionine labelled components of *P. berghei* infected erythrocytes (tracks 4, 5, 6).
F. Immunological and Biochemical Characteristics of Membranous and Soluble Components of Three Times Freeze-Thawed *Plasmodium berghei* Infected Erythrocytes and of Soluble Material Washed From the Surface of Free Plasmodia.

Figure 1 shows the parasitemias after challenge in rats vaccinated with various fractions of 3x freeze-thawed *P. berghei* parasites. Vaccination with unfractionated parasites gave the best protection. The soluble and membranous fractions also stimulated good protection but somewhat less than did the unfractionated parasites. The control preparation of uninfected erythrocyte membranes stimulated the least amount of protection. One dose of unfractionated parasites or uninfected erythrocyte membranes contained 1294 ± 147 ug protein. One dose of the soluble fraction contained 472 ± 98 ug of protein and one dose of the membranous fraction contained 944 ± 188 ug of protein. Results of this study and those of a previous study (Saul and Kreier, 1977) indicate that an immunogen inducing a protective immune response was present in both the membranous and soluble fraction of 3x freeze-thawed parasites.

Figure 20 shows autoradiographs and Coomassie blue stains of a 10% acrylamide gel containing samples of membranous and soluble fractions of *P. berghei* infected erythrocytes. These fractions were prepared by procedures similar to those used in the immunization study. The autoradiographs of the unfractionated, infected erythrocytes (track 3) and the membranous fraction (track 2) were qualitatively similar. The soluble fraction (track 1) differed from the other fractions in
Figure 19. Parasitemia after challenge in rats vaccinated with unfractionated *P. berghei* free parasites (FP) and with soluble and insoluble fractions of three times freeze-thawed *P. berghei* free parasites (FP). Each point is an average derived from the four rats in each group. Saponin was used as an adjuvant and uninfected erythrocyte membranes were used as a control vaccine.
FIGURE 19

# OF INFECTED ERYTHROCYTES/10,000 ERYTHROCYTES

- RBC MEMBRANES AND SAPONIN
- F.P. SOLUBLE AND SAPONIN
- F.P. INSOLUBLE AND SAPONIN
- F.P. TOTAL AND SAPONIN

DAYS AFTER CHALLENGE
Figure 20. Components of soluble and membranous fractions of three times freeze-thawed *P. berghei* infected erythrocytes after electrophoresis in 10% polyacrylamide gel. Tracks 1-4, autoradiographs; tracks 5-7, Coomassie blue stains; track 1, soluble fraction; track 2, membranous fraction; track 3, unfractionated infected preparation; track 4, unfractionated uninfected preparation (control); track 5, unfractionated infected; track 6, membranous fraction; track 7, soluble fraction.
FIGURE 20

MWT X 1000

1 2 3 4 5 6 7

92 ▶ 66 ▶ 45 ▶ 31 ▶ 21 ▶
that it lacked the higher molecular weight components (90,000 daltons). The Coomassie blue stained components of the unfractionated infected erythrocytes (track 5), the soluble fraction (track 6) and the membranous fraction (track 7) were similar. The membranous fraction was almost totally lacking in components having molecular weights of less than 50,000 daltons. The soluble fraction appeared to have less dense bands in the very high molecular weight range.

Figure 21 shows the parasitemias after challenge in rats which had been immunized with various preparations of P. berghei free parasites. The unwashed free parasite vaccine stimulated the best protection. The washed parasites and the material washed from the surface of free parasites ("washoff antigen") stimulated slightly less protection but still fairly strong protection. The uninfected erythrocyte membrane (control) stimulated the least amount of protection. One dose of washed or unwashed free parasites contained 1294 ± 147 ug of protein; one dose of "washoff antigen" contained 70 ± 20 ug of protein (Grothaus and Kreier, 1980).

Sera from normal (unimmunized) rabbits and sera from rabbits which had been immunized against the various preparations used in the rat immunization studies were allowed to react with solubilized preparations of 35S-methionine labelled antigens. Immune complexes were collected using a suspension of Protein A bearing Staph. aureus. Immune complexes were then dissociated and electrophoresed on polyacrylamide gels. Autoradiographs of acrylamide gels characterizing the labelled plasmodial components which reacted with the various serum samples were prepared.
Figure 21. Parasitemias after challenge in rats vaccinated with unwashed *P. berghei* free parasites (FP), washed free parasites (FP) and material washed from the surface of free parasites (washoff antigen). Saponin was used as adjuvant and uninfected rat erythrocyte membranes were used as a control vaccine. Each point is an average derived from the four rats in the group.
FIGURE 21

# OF INFECTED ERYTHROCYTES/10,000 ERYTHROCYTES

- ■ RBC
- ○ WASHED F.P.
- □ WASHOFF AG
- ● UNWASHED F.P.

DAYS AFTER CHALLENGE
The unreacted labelled plasmodial preparations contained a total of about 30 components (Figure 22, track 5; Figure 23, track 19). There were only very small amounts of the plasmodial components absorbed onto the Staph. aureus in the absence of serum (Figure 22, track 13). None of the components of $^{35}$S-methionine labelled uninfected erythrocytes reacted with anti-free parasite serum (Figure 22, tracks 6 and 7) or with anti-host serum (Figure 22, tracks 8 and 9).

Serum from rabbits immunized against uninfected rat erythrocytes had relatively weak reactions with 8-10 of the larger molecular weight components of the labelled plasmodia (Fig. 22, tracks 1 and 2; Fig. 23, tracks 8, 9, 18; Fig. 24, track 2). The anti-host serum did not react with plasmodial components having molecular weights of less than 40,000 daltons.

Serum from rabbits immunized against P. berghei free parasites reacted with a greater number of plasmodial components than did the anti-host serum and showed a very strong reaction with several of the components (Fig. 22, tracks 1 and 2; Fig. 23, tracks 6, 7, 14, 15, 16, 17; Fig. 24, track 3). Plasmodial components having molecular weights of 78,000, 60,000 and 44,000 reacted with anti-free parasite serum but did not react with anti-host serum. Four plasmodial components having molecular weights of 25,000, 28,000, 30,000, and 35,000 appeared as very dark bands indicating a very intense reaction with the anti-free parasite serum. These same small molecular weight plasmodial components (between 25,000 and 35,000) also reacted intensely with serum from rabbits which had been immunized against "washoff antigen"
Figure 22. Autoradiographs of a 10% acrylamide gel showing the labelled plasmodial components which reacted with serum from rabbits immunized against *P. berghei* free parasites (tracks 1 and 2) or uninfected rat erythrocytes (tracks 3 and 4). Track 5 is the unreacted S-methionine labelled plasmodial preparation. Track 13 would show any labelled plasmodial components non-specifically absorbed by Protein A bearing *S. aureus* in the absence of serum. Serum samples from rabbits immunized against *P. berghei* free parasites (tracks 6 and 7) and uninfected rat erythrocytes (tracks 8 and 9) were reacted with a preparation of 35S-methionine labelled uninfected erythrocytes. Autoradiograph A was exposed for 20 hours; B was exposed for 4 days and C was exposed for 10 days.
Figure 23. Autoradiographs of 10% acrylamide gel showing the labelled plasmodial components which reacted with serum from rabbits immunized against different preparations of P. berghei free parasites (tracks 6, 7, 14, 15, 16, 17), uninfected erythrocytes (tracks 8, 9, 18), or material washed from the surface of free parasites ("washoff antigen") (tracks 12 and 13). Track 19 is a sample of the total $^{35}S$-methionine labelled plasmodial preparation. Autoradiograph A was exposed for 48 hours and autoradiograph B was exposed for 7 days.
Figure 24. Autoradiograph of a 10% acrylamide gel showing the labelled plasmodial components which reacted with normal (uninfected) rabbit serum (track 1) and serum from rabbits immunized with uninfected rat erythrocytes (track 2), P. berghei free parasites (track 3) or "washoff" antigen.
(Figure 23, tracks 12 and 13; Figure 24, track 4). The anti-washoff serum reacted very weakly with the higher molecular weight plasmodial components.

Normal (unimmunized) rabbit serum showed a weak reactivity with 5 of the higher molecular weight plasmodial components (Figure 24, track 1).

G. Characterization of the Components of *Plasmodium berghei* Infected Erythrocytes by Immunoprecipitation with Serum From Rats Collected During the Course of Infection with *Plasmodium berghei*.

Figure 25 is an autoradiograph of a 10% polyacrylamide gel in which the labelled plasmodial components which reacted with rat serum collected during the course of infection with *P. berghei* are resolved. Preinfection (normal) rat serum reacted weakly with 5 of the higher molecular weight plasmodial components (track 1). These components had molecular weights of approximately 100,000, 65,000, 60,000, 45,000 and 40,000 daltons. Serum collected 5 days after infection when the parasitemia was 1% had only a very weak reaction with the same components (track 2). At 14 days after infection when the parasitemia was 31%, the serum reacted with two additional plasmodial components which had molecular weights of 77,000 and 25,000 daltons. The reaction with the 25,000 molecular weight component was particularly intense (track 3). Serum collected 19 days after infection when the parasitemia was 91% appeared to have an even stronger reaction to the 77,000 and 25,000 molecular weight components (track 4). At 23 days post
Figure 25. Autoradiograph of a 10% acrylamide gel characterizing the labelled plasmodial components which reacted with the serum from rats taken at various times during the course of infection with *P. berghei*. Track 1, preinfection (normal) rat serum; track 2, 5 days post infection (1% parasitemia); track 3, 14 days post infection (31% parasitemia); track 4, 19 days post infection (90% parasitemia); Track 5, positive control-unreacted labelled plasmodial antigen; Track 6, 23 days post infection (recovered rat, no parasitemia); track 7, serum from a rat which had recovered sometime earlier; Track 10, negative control-components which bound to Staphlococcal Protein A in the absence of serum.
infection, when the parasitemia had just been cleared, the serum reacted with the same set of plasmodial components (track 6). Serum from a rat which had recovered from infection sometime earlier did not seem to have quite as strong a reaction with the 25,000 and 77,000 molecular weight components (track 7). Track 10 is the negative control showing the labelled plasmodial components which bound to the Staphylococcal Protein A in the absence of serum. Two very faint bands (molecular weight 60,000 and 65,000) indicated that a very small amount of these plasmodial components may have been bound by the Staph. Protein A.
DISCUSSION

The identification of plasmodial components through the use of conventional protein staining techniques after the components have been electrophoresed on polyacrylamide gels is complicated by the presence of host erythrocyte components contaminating the preparations. Although the identification of more than 10 plasmodial proteins has been reported using non-SDS polyacrylamide gel electrophoresis (PAGE) (Corradetti et al., 1971; Finerty and Dimopoullus, 1968; Sodeman and Neuwissen, 1966; Chavin, 1966; Spira and Zuckermann, 1966; Kreier et al., 1976), it is now known that most of these preparations contained at least some contaminating erythrocyte materials (Kreier, 1977). Since comparisons with the patterns of appropriate host erythrocyte membrane controls were not included in most of these studies, it is not possible to determine which of the proteins were host and which were plasmodial.

The infected and uninfected rat erythrocyte membrane preparations in our study had proteins with molecular weights similar to 5 of the 6 major proteins contained in normal human erythrocyte membranes (Fairbanks et al., 1971; Marchesi and Furthmayer, 1976) (Figure 1). Both of our erythrocyte membrane preparations also contained two other major proteins with molecular weights of 140,000 and 62,000 daltons. We also observed lesser amounts of a few other small molecular weight proteins in the erythrocyte membrane preparations. These may represent proteolytic cleavage products of the major membrane proteins or possibly proteins which are present in rat erythrocyte membranes but
not in human erythrocyte membranes.

Erythrocyte membrane contamination in our free parasite preparation is indicated by the presence of membrane proteins I and II (spectrin, molecular weights 225,000 and 250,000) and lesser amounts of membrane proteins III (89,000), IV (77,500) and V (41,000). Hemoglobin, mostly in the form of a 32,000 molecular weight subunit, was also present in the free parasite preparation and to a lesser degree in the erythrocyte membrane preparations.

We were able to consistently detect one component having a molecular weight of approximately 52,000 which appeared in all plasmodial preparations (infected membranes and free parasites) but did not appear in the uninfected erythrocyte membrane preparations. It is possible that this 52,000 molecular weight component is the product of proteolytic action of plasmodial enzymes on erythrocyte membrane proteins. Yutnavong (1979), however, did not report the appearance of any new bands when normal erythrocyte membranes were analyzed after they were incubated in the presence of preparations of soluble P. berghei.

Some workers have reported a significant decrease in the quantity of spectrin (protein bands I and II) in the erythrocyte membranes of parasitized cells (Weidecam et al., 1973; König and Mirsch, 1977; Sherman and Jones, 1979). Others have not observed this decrease in spectrin upon examination of parasitized erythrocyte membranes (Eisen, 1977; Deas, 1981). PAS stains by these workers were similar in that they showed only variable quantitative differences but no qualitative differences in the patterns of infected or uninfected erythrocyte
membranes. We did not attempt a quantitative analysis of our results, however observations indicated no extreme difference in the intensity of spectrin bands of infected or uninfected membranes.

Some parasite associated components in the 150,000 – 165,000 molecular weight range have been identified in erythrocyte membranes infected with *P. berghei* (Weidecam *et al.*, 1973; Yuthavong *et al.*, 1979) and *P. chabaudi* (Konigk and Mirsch, 1977).

The silver-based color stain was also used to detect proteins in *P. berghei* infected and uninfected erythrocyte membranes and free parasites (Figure 2). The amount of protein in the samples analyzed on this gel was only about 1/10 the amount of protein on the gel stained by Coomassie blue in Figure 1. The high sensitivity of detection on this gel enabled us to detect 3 components which were unique to the plasmodial preparations. They had molecular weights of 115,000, 57,000 and 28,000 daltons. The component having a molecular weight of 57,000 appears as a double band on this gel and is probably the same component which we identified on the Coomassie blue stain as having a molecular weight of 52,000.

The appearance of a greater number of bands and the increased resolution using a reduced amount of protein illustrates the greater sensitivity of this technique as compared to the more conventional Coomassie blue staining technique. The silver stain detects an amount of protein comparable to that detectable by autoradiography of $^{35}$S-methionine labelled proteins (Sammons *et al.*, 1980). This technique may be useful in the further identification of plasmodial components in situations where the use of radioisotopes is not desirable.
It was possible to identify only a few plasmodial components by using conventional staining techniques. The presence of many host related components was demonstrated by the staining of our infected membranes and free parasites. We therefore undertook a study to identify the components of \textit{P. berghei} through the incorporation of radiolabelled precursors.

Our results showed that $^3$H-uridine was incorporated by \textit{P. berghei} infected erythrocytes. We did not observe incorporation of $^3$H-thymidine by \textit{P. berghei} infected erythrocytes (Table I; Figure 3). Most previous studies have shown that purines are transported into malaria infected erythrocytes but pyrimidines are not incorporated. Bungen and Neilson (1969) reported that cytidine and uridine were not incorporated by \textit{P. berghei} infected erythrocytes. Conklin (1973) however reported limited utilization of thymidine and uridine by \textit{P. knowlesi} infected erythrocytes. Neame and Homewood showed that \textit{P. berghei} infected cells at least accomplished equilibrium of cytidine and uridine across the erythrocyte membrane. It has been suggested that these pyrimidines were not actually incorporated into the nucleic acids of the parasite. The parasite probably has the necessary enzymes needed to synthesize its own pyrimidines de novo or by salvage mechanisms.

Although our results do indicate that $^3$H-uridine was incorporated by \textit{P. berghei} infected erythrocytes, we did not attempt to isolate parasite nucleic acids or further pursue the labelling of nucleic acids. Very little work has been done in this area and this type of investigation would probably yield useful information about the malaria
3H-glucosamine was used in this study to label glycoproteins. The surface components of many cells contain glycoproteins and we postulate that the merozoite surface coat may also contain glycoproteins.

Our results indicate that *P. berghei* infected erythrocytes incorporated 3H-glucosamine (Table II; Figure 4). The glucosamine was incorporated into at least two glycoproteins by the infected erythrocytes (Figure 5). The smaller of these components had a molecular weight of about 50,000 daltons and the larger had a molecular weight in excess of 200,000 daltons. There was also a substantial amount of labelled material which did not enter the separation gel. We did not detect any 3H-glucosamine labelled components in the uninfected erythrocyte preparations (Figure 5).

Although no other studies have been reported of 3H-glucosamine incorporation by rodent plasmodia, 3H-glucosamine was incorporated into between 6 and 8 glycoproteins by *P. falciparum* infected erythrocytes (Kilejian, 1980; Udeinya and Van Dyke, 1980, 1981). One of the major glycoproteins detected by Kilejian (1980) is nearly identical in relative mobility to the larger of the two glycoproteins (200,000 Mwt.) identified in this study. The metabolic labelling procedure used in our study does not determine whether the labelled glycoprotein is located on the erythrocyte surface or on the intracellular parasite. A large molecular weight glycoprotein is an excellent candidate for the merozoite surface coat.

Although many amino acids may be obtained by plasmodial digestion of erythrocyte hemoglobin, most free amino acids are readily
incorporated by plasmodia in infected cells. Various plasmodia in infected cells have been shown to incorporate arginine, lysine, leucine, proline, histidine (Sherman, 1977), methionine, isoleucine and cysteine (McCormik, 1970). The incorporation of \( ^3 \)H-isoleucine (McColm et al., 1980), \(^{35}\)S-methionine (Tait, 1981) and \(^3\)H-leucine (Knopf et al., 1979) have been used to metabolically label plasmodial components for further analysis.

Our results indicated that \(^{35}\)S-methionine was incorporated by \( P. \) berghei infected erythrocytes (Table 3; Figure 6). The \(^{35}\)S-methionine was incorporated into between 25 and 30 components by the infected erythrocytes (Figure 7). Reticulocyte rich (approximately 50%) populations of uninfected erythrocytes were induced by injecting phenylhydrazine into normal rats. Reticulocytes synthesized hemoglobin and the uninfected samples also contained a significant number of labelled smaller molecular weight components which migrated with the tracking dye (Figure 7). Since \( P. \) berghei infection of rodents causes a high reticulocytosis, it is important that reticulocyte-rich populations of uninfected erythrocytes be included as controls in all metabolic uptake erythrocytes.

Others have identified parasite related components released into the culture supernatants (SN) of \( P. \) falciparum (Wilson and Bartholemew, 1975), \( P. \) knowlesi (McColm et al., 1977) and \( P. \) berghei (Weissberger et al., 1979) infected erythrocytes. These components have been shown to stimulate lymphocyte transformation of non-adherent spleen lymphocytes (Weissberger et al., 1975).
We were not able to identify any parasite related materials in the culture supernatants during this study. The number of trichloroacetic acid (TCA) precipitable counts in the culture supernatants from 3H-glucosamine-labelled infected and uninfected supernatants were similar (Table 2; Figure 5). While there was an increase in the number of TCA precipitable counts in the culture supernatants of 35S-methionine labelled infected erythrocytes (Table 3; Figure 6), we did not detect any labelled component unique to the infected cultures upon polyacrylamide gel analysis of the culture supernatants (Figure 13). McCollm et al. (1977) observed that the release of these materials occurred during the period of schizont rupture and merozoite reinvasion of erythrocytes. Our system probably did not support the reinvasion of erythrocytes (see below) and material released at this point in the life cycle would not have been detected in our culture system.

The 35S-methionine labelled components were chosen for the most detailed identification and characterization presented in this work. The uptake of 3H-uridine and 3H-thymidine by infected erythrocytes was not clearly defined and the parasite nucleic acids would not be suitable for our purposes. The electrons emitted from the 35S-methionine are of a much higher energy that those emitted from 3H-glucosamine and are much easier to detect so the autoradiographs are exposed in a much shorter time. Additionally, there were a much larger number of 35S-methionine labelled components detectable by PAGE analysis. The glycoproteins labelled with 3H-glucosamine would probably also be identified through labelling of the polypeptide component with 35S-methionine. The further analysis of 3H-glucosamine
labelled plasmodial components should not be ignored.

In our initial uptake experiments, erythrocytes were continuously exposed to $^{35}$S-methionine for up to 72 hours. We found that after about 24 hours, our ability to collect a pellet of erythrocytes was affected by lysis of the erythrocytes. The number of counts incorporated (Table 3; Figure 6) and polyacrylamide gel analysis (Figure 7) indicated that there was probably little additional incorporation of $^{35}$S-methionine after the first 5 hours in culture. We had not been able to establish the reinvagination of erythrocytes by P. berghei in culture using the candle jar method of Trager (Birmingham, Master's Thesis, OSU, 1980). In this study, we used Temin's Modified MEM instead of RPMI 1640 in order to reduce the amount of competition with cold methionine in the culture medium. It was necessary to establish the amount of time in culture which gave the maximum amount of $^{35}$S-methionine incorporation with the least amount of erythrocyte lysis.

The counts from the pulse label experiment showed that almost 80% of the total label incorporated by infected erythrocytes was incorporated during the first 5 hours in culture (Table IV; Figure 8). Giemsa smears from infected cultures showed only slight maturation of intracellular parasites during the course of the experiment. The erythrocytes began to deteriorate rapidly after the first 10-15 hours and few intact cells were present after 24 hours. The infected erythrocyte cultures clearly deteriorated more rapidly than the uninfected cultures. Infectivity of the parasites was determined by injecting 0.4 mls of the culture contents into Swiss mice after various
times in culture (data not shown). The infectivity of the parasites declined rapidly over the first 20 hours in culture and no mice showed patent parasitemia after being injected with infected erythrocytes which had been cultured in excess of 20 hours.

The polyacrylamide gel showing samples from the pulse label (Figure 9) also shows that the majority of the label was incorporated during the first 5 hours exposure. Although a much smaller amount of $^{35}$S-methionine was incorporated during the second five hour period (Figure 9b, track 2), a very long exposure of this gel showed that the labelled polypeptides were identical to those labelled during the first 5 hour period (Figure 9a, track 1).

We conclude from these analyses that 5-10 hours exposure to $^{35}$S-methionine was sufficient to label the plasmodial components for use in this study.

The identification of plasmodial components was continued by electrophoresis of the various samples on a series of polyacrylamide gels (Figure 10, 7.5% acrylamide; Figures 11 and 12, 10-20% acrylamide gradients). We were able to detect only one plasmodial component by Coomassie blue staining of infected erythrocytes. Autoradiographs of the samples confirmed the presence of 25-30 labelled components which appeared consistently in the infected samples but did not appear in the uninfected samples. The few components which were labelled by uninfected populations of reticulocyte-rich erythrocytes seemed to be hemoglobin, hemoglobin dimer subunits or small molecular weight components which did not resolve on the gel. Some of the small components are probably hemoglobin monomers however any incomplete or
small polypeptide chains which were not yet incorporated into a larger protein would also migrate with the tracking dye. Additionally, these small molecular weight components could represent breakdown products of larger proteins.

Leukocytes were removed by filtration through packed cellulose powder according to the procedure of Fulton and Grant (1956). This procedure was found to remove all leukocytes from *P. berghei* infected rat and mouse blood (Housewood and Neame, 1976). The presence of even a very small percentage of contaminating leukocytes in a metabolic labelling procedure such as this would invalidate the results. It is probable that the large number of components labelled with $^{35}$S-methionine in the infected erythrocytes were *P. berghei* components, unless parasitization somehow caused the recommencement of erythrocytic protein synthesis which is unlikely.

The reproducibility of our system was tested by comparing the labelled plasmodial components obtained from four separate experiments on a single gel (Figure 14). Since *P. berghei* causes asynchronous infection in rats, the relative proportions of rings, trophozoites and schizonts could vary considerably between populations of 20% parasitized cells. Infected blood, harvested from four different rats and labelled during the course of four separate experiments was analyzed. We determined that although the total amount of radioactive incorporation varied somewhat from sample to sample, the number, intensity and mobility of the labelled components in the four samples were nearly identical.
We were able to identify approximately 32 $^{35}$S-methionine labelled plasmodial components in our samples (Figure 15). The molecular weights ranged from 18,000 to greater than 200,000 daltons. These molecular weights were determined by comparison with the mobility of a series of known molecular weight standards which were analyzed under identical conditions (see Appendix II).

The components of *P. falciparum* (Perrin *et al.*, 1980; Tait, 1981; Kilejian, 1980) and *P. knowlesi* (McColm *et al.*, 1980) have been identified using metabolic labelling with amino acids. Tait (1981) identified 35 $^{35}$S-methionine labelled components and Kilejian (1980) identified about 30 $^3$H-proline labelled components of *P. falciparum*. A majority of these labelled plasmodial components had relative intensities and mobilities similar to the components identified in this study. Knopf *et al.* (1979) was able to identify 15 $^3$H-leucine labelled components in a similar study using *P. berghei*. Although we did use PMSF to minimize the action of proteolytic enzymes, it is possible that some of our components could be the result of proteolytic cleavage of larger proteins. Also, there may be differences in the levels of incorporation of various amino acids into parasite proteins.

The components of *P. falciparum* infected human erythrocytes were also identified in this study. We compared the amount of $^{35}$S-methionine incorporation by *P. falciparum* infected erythrocytes cultured in RPMI 1640 or Temin's Modified MEM. Any advantage in the uptake of labelled methionine which might have been gained by the reduction of competition with cold methionine was more than overcome by the decrease in incorporation resulting from the poor conditions in the
cultures containing the MEM (Table 5; Figure 16). The infected erythrocytes incorporated more $^{35}$S-methionine than the uninfected human erythrocytes regardless of the culture medium in which they were grown (Table 5; Figure 16). We were able to identify about 24 labelled components of *P. falciparum* which were not detected in samples of uninfected human erythrocytes using polyacrylamide gel electrophoresis (Figure 17).

We observed many similarities and at least one major difference in comparing the $^{35}$S-methionine labelled components of *P. berghei* to those of *P. falciparum* (Figure 18). The major component of *P. falciparum* which was completely absent from *P. berghei* had a molecular weight of 160,000 daltons. Wallach and Conley (1977) identified a 165,000 molecular weight component in *P. knowlesi* infected erythrocytes by I-lactoperoxidase surface labelling. The other labelled components appeared in similar concentrations in both *P. berghei* and *P. falciparum* infected erythrocytes. Similarities and differences in the labelling characteristics of the various plasmodial species will have to be defined if information derived from experimental systems is to be applied to human malaria.

*P. berghei* free parasites were freeze-thawed three times and separated into soluble and insoluble (membranous) fractions by centrifugation at 30,000 x g for 30 minutes. The immunogenicity of the two fractions was evaluated by vaccination of rats and the biochemical composition of each fraction was analyzed by SDS PAGE.
The protection stimulating antigen was partitioned between the soluble and membranous fractions of three times freeze-thawed free parasites (Figure 19). The soluble fraction contained $472 \pm 98$ ug of protein per dose and the membranous fraction contained $944 \pm 118$ ug of protein per dose. The soluble portion contained somewhat less total protein than the membranous fraction. Since these two fractions were roughly equivalent in immunogenicity, this implies that the soluble fraction may be somewhat richer in protective antigen. Similar results were obtained in a previous evaluation of the immunogenicity of soluble and membranous fractions of *P. berghei* (Saul and Kreier, 1977).

The soluble and membranous fractions of three-times freeze-thawed free parasites were analyzed by electrophoresis on polyacrylamide gels (Figure 20). Coomassie blue stains of the fractions and unfractionated samples showed that there was a reduction in the amount of small molecular weight components in the insoluble fraction (less than 50,000 MWt.).

Autoradiography of the labelled plasmodial components showed that the soluble fraction was almost totally lacking in components having molecular weights greater than 90,000. The membranous fraction contained a significant amount of the smaller molecular weight soluble components. This was probably due to insufficient washing of the membranous pellet.

The results of PAGE analysis considered along with the immunization studies indicate that some of the middle and lower weight components are likely to be important immunogens. If freeze-thawing dislodges only some of the antigenic components from the membrane, then
antigens capable of stimulating a protective immune response may in fact be membrane associated and still appear in the soluble fraction of freeze-thawed parasites.

Rats were also immunized with material washed from the surface of free parasites (Figure 21). The washed free parasites and washings from the parasites gave slightly less protection than the unwashed parasites, but still gave fairly strong protection. It is an important point that the amount of protein in a single dose of washoff antigen (70 ± 20 ug) was much less than the amount of protein contained in a dose of washed or unwashed parasites (1294 ± 147 ug). The fact that an antigen fraction containing only 1/20 of the parasite protein stimulates almost as much protection as does the whole parasite suggests that the washoff antigen may be a concentrated preparation of the protection stimulating immunogen.

Serum from rabbits immunized against various plasmodial preparations and against host (rat) erythrocytes was reacted with preparations of solubilized, $^{35}$S-methionine labelled plasmodial components. Figures 22 and 23 are autoradiographs showing the labelled plasmodial components which reacted with anti-host, anti-free parasite and anti-washoff serum samples produced in a number of different rabbits. Figure 24 shows representative samples of the plasmodial components which reacted with each serum sample for easier comparison.

Anti-host serum reacted to some degree with a number of the labelled plasmodial components. The components which were bound by anti-host serum were of a middle to high molecular weight and showed only a minimal reactivity. Normal rabbit serum reacted with a similar
set of plasmodial components. There may be constitutive antibodies in normal rabbit serum which react to some degree with plasmodial antigens. Alternatively, these components may be present due to non-specific binding of labelled plasmodial components to the Staphylococcal Protein A. Figure 23C, track 13 shows the labelled plasmodial components which bound to Staphylococal Protein A in the absence of serum. A very long (10 day) exposure of the gel shows a very small amount of labelled materials similar in relative mobilities to the components which appeared to react with anti-host and normal rabbit serum.

The anti-free parasite and anti-washoff serum bound a large number of plasmodial components. These antibodies apparently had a strong affinity for a number of the plasmodial components. Seven components having molecular weights ranging from 25,000 to 100,000 daltons were bound by the anti-plasmodial serum which did not react with the anti-host serum (Figures 22, 23 and 24). The great intensities of the bands produced by the components in the 25,000 to 35,000 molecular weight range indicate that they had very strong reactivity with the anti-plasmodial antibodies. Anti-washoff antibodies very strongly bound the smaller molecular weight components but did not react as strongly with the higher molecular weight components.

The rabbit is not a natural host of *P. berghei* and therefore the production of antibodies to these components is not necessarily an indication of a protective immunogen. We were able to show that an identical preparation of the washoff antigen was a potent immunogen in our rat vaccination study (Figure 21) and the strong antigenicity of
these low molecular weight components in an artificial host is probably significant.

Serum samples from rats collected at various times during the course of infection with *P. berghei* were also reacted with our preparation of labelled plasmodial components (Figure 25). Preinfection rat serum bound components similar to those which were bound by normal rabbit serum. These may be components which were non-specifically bound to the Staphyloccal Protein A but probably represent material bound by constitutive antibodies in normal rat serum which reacted weakly with plasmodial components. Serum collected 5 days after infection appeared to be depleted of constitutive antibodies. The constitutive antibodies may have been bound by parasite components found in the blood of the infected rat. Serum samples collected 14 days after infection (parasitemia 31%) bound two plasmodial components for which no constitutive antibodies had existed. These plasmodial components had molecular weights of 77,000 and 25,000 daltons.

Serum samples collected from rats 19 days after infection and from a recently recovered rat also had a very high affinity for these same two plasmodial components. The reaction of the induced antibodies was extremely strong to the plasmodial component having the smaller molecular weight (25,000 daltons). The plasmodial components which reacted with constituitive antibodies are not likely to be candidates for major protective antigens. Components which reacted with antibodies which were induced by infection are much better candidates.
The rat is a natural host of *P. berghei* and generally mounts enough of an immune response to recover from infection. We did not actually correlate the appearance of antibodies having affinity for the 25,000 Mwt. component with the onset of immunity in this experiment. We are currently collecting serum samples from a much larger pool of infected rats. We will attempt to correlate the appearance of antibodies with the ability of serum to passively protect mice from infection with *P. berghei*.

We have presented strong evidence that the major protective antigen for *P. berghei* is a 25-35,000 dalton component which can be washed from the surface of the parasite. The material washed from the surface of free parasites was approximately 20 times as potent an immunogen as were the whole free parasites. Serum from rabbits immunized against the "washoff antigen" had a very high affinity for plasmodial components having molecular weights between 25,000 and 35,000 daltons. Infection of rats with *P. berghei* also appeared to induce the formation of an antibody with a very strong affinity for a similar plasmodial component of 25,000 daltons.

Recently, other researchers have used hybridoma technology to produce monoclonal antibodies which react with only one or two components of various species of malaria parasites. Epstein et al. (1981) produced monoclonal antibodies which agglutinated merozoites of *P. knowlesi* and blocked merozoite reinvasion of erythrocytes. The antigen which reacted with this monoclonal antibody had a molecular weight of about 250,000 daltons. A monoclonal antibody against a 235,000 dalton merozoite antigen of *P. yoelii* was capable of passively
protecting mice from infection with the parasite (Freeman et al., 1980; Holder and Freeman, 1981). A similar monoclonal antibody to P. yoelii merozoites was produced by Taylor et al. (1981), although the molecular weight of the corresponding antigen has not yet been determined. Perrin et al. (1980) initially produced a monoclonal antibody to a 195,000 molecular weight component of P. falciparum. This monoclonal antibody did not inhibit growth of P. falciparum in vitro. The same group raised monoclonal antibodies to antigens having molecular weights of 36,000 and 95,000 daltons which did inhibit in vitro growth of P. falciparum.

All of the protective antigens identified in these studies have much higher molecular weights than the 25,000 dalton "washoff antigen" identified in our study. There does seem to be agreement that the protective antigens identified in all of these studies are merozoite associated.

We propose the following hypothesis to explain the wide range of molecular weights of the various antigens which have been discussed as vaccine candidates. The merozoite membrane contains a glycoprotein having a molecular weight in the vicinity of 250,000 daltons. A large portion of this molecule may be imbedded in the phospholipid bilayer of the membrane. The imbedded portion of the molecule probably contains very few of the carbohydrate residues of the glycoprotein but accounts for a relatively large percentage of the molecular weight. This portion of the molecule would be hydrophobic but can probably be fully extracted by treatment with detergents.
A smaller but extremely important part of the molecule is probably exposed on the external side of the plasma membrane. This portion of the glycoprotein contains a majority of the carbohydrate residues but only accounts for a small percentage of the molecular weight (i.e. 25,000 - 35,000 daltons). We believe that this portion of the molecule is the major component of the surface coat of the merozoite. This hypothesis is summarized in Figure 26.

A significant amount of the carbohydrate containing portion (surface coat) is apparently shed by the merozoite or can be separated from the surface of merozoites by relatively mild physical forces. Parasite components having molecular weights around 40,000 daltons have been isolated from the culture supernatants of \textit{P. knowlesi} infected cells (McColm \textit{et al.}, 1977). James \textit{et al.}, (1981) have successfully isolated the protective antigens from a closely related intracellular parasite (\textit{Babesia bovis}) from culture supernatants. These components had molecular weights of 37,000 to 40,000 daltons.

The procedure used to obtain "washoff antigen" would yield an extremely rich preparation of the surface coat material. Freeze-thawing would also probably release a significant amount of the surface coat material into the soluble material. This would account for the partitioning of immunoenicity between the soluble and membraneous fractions of the freeze-thawed free parasites.

Whether the large molecular weight components (total glycoprotein molecule) or the small molecular weight component (external portion only) was identified by immunoprecipitation is probably related to differences in the extraction and solubilization procedures employed
Figure 26. Merozoite Membrane Model.
Figure 86.

Hydrophilic region extends beyond surface antigenic determinants for protective antigen.

Hydrophobic region imbedded in bilayer, few -CHO residues, carrier protein.

25-30,000 d cleaved here.

Surface coat material (Washoff antigen).

Phospholipid bilayer.
during the preparation of the radiolabelled plasmodial antigens.

Simple exposure of the labelled parasites to detergents is probably an effective technique for extraction of the entire glycoprotein molecule from the merozoite membrane. This is the method of antigen preparation used by Perrin et al. (1980) and Holder and Freeman, (1981). This type of extraction was performed in the presence of as many as 5 protease inhibitors (Epstein et al., 1981) which would be further assurance that the integrity of the entire molecule would be preserved. The entire glycoprotein would then be immunoprecipitated by antibody to the relatively small antigenic determinant.

In addition to extraction with detergents, our radiolabelled plasmodial preparations were sonicated before they were reacted with the serum samples. Although PMSF was present as a protease inhibitor, our preparations may have been susceptible to proteolytic degradation. The smaller molecular weight portion of the molecule containing the important antigenic determinants was apparently separated from the larger molecular weight carrier portion in our preparations. It was therefore precipitated in the 25,000 to 35,000 form by the serum.
SUMMARY

Some components of *Plasmodium berghei* in mixtures of host erythrocytes and *Plasmodium berghei* were identified by electrophoresis in polyacrylamide gels. One component (52,000 molecular weight) present in the plasmodia-host mixture but not present in host erythrocyte preparations was identified by Coomassie blue staining and 3 such components (molecular weights of 28,000, 57,000 and 115,000) were identified by silver based protein staining of the gels.

In vitro, *Plasmodium berghei* infected erythrocytes incorporated more $^{35}$S-methionine, $^3$H-glucosamine and $^3$H-uridine but not more $^3$H-thymidine than did uninfected rat erythrocytes. Autoradiography of polyacrylamide gels containing preparations of cells infected with plasmodia showed that the $^3$H-glucosamine was incorporated into two components with molecular weights of 50,000 and 200,000 and $^{35}$S-methionine was incorporated into approximately 32 polypeptides with molecular weights ranging from 18,000 to 200,000. Only hemoglobin and some small molecular weight materials were labelled by uninfected erythrocytes.

Many similarities and one difference was observed when $^{35}$S-methionine labelled polypeptides of *Plasmodium berghei* were compared to $^{35}$S-methionine labelled polypeptides of *Plasmodium falciparum*.

Both soluble and insoluble fractions of three times freeze-thawed *Plasmodium berghei* were capable of inducing immunity in rats. $^{35}$S-methionine labelled polypeptides derived from whole plasmodia and soluble and insoluble fractions of plasmodia differed in that the soluble preparations lacked the polypeptides having molecular weights
greater than 90,000.

Soluble material which was obtained by gentle washing of the free parasite preparations induced the highest degree of immunity in rats.

Serum from rabbits immunized with the various plasmodial fractions was used to precipitate $^{35}$S-methionine labelled preparations of Plasmodium berghei. Rabbit antisera to the soluble preparations which induced the highest degree of immunity in rats reacted with a variety of plasmodial components but most strongly with plasmodial components having molecular weights of 24,000, 30,000 and 35,000.

Serum from rats collected during the course of infection with Plasmodium berghei was also used to precipitate $^{35}$S-methionine labelled preparations of Plasmodium berghei. Serum collected from rats 14 days post-infection and serum from recovered rats reacted with 9 plasmodial components but reacted most strongly with a component having a molecular weight of 25,000. Serum from normal rats and serum collected from rats 5 days post-infection did not react with the 25,000 molecular weight component.
APPENDIX I: Tables
Table 1. Dynamics of $^3$H-thymidine and $^3$H-uridine incorporation by *P. berghei* infected and uninfected erythrocytes.

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>$^3$H-thymidine Incorporation</th>
<th>$^3$H-uridine Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected Reticulocyte-rich Population</td>
<td>Infected Reticulocyte-rich Population</td>
</tr>
<tr>
<td></td>
<td>Uninfected Reticulocyte-rich Population</td>
<td>Uninfected Reticulocyte-rich Population</td>
</tr>
<tr>
<td></td>
<td>1  2  Ave.</td>
<td>1  2  Ave.</td>
</tr>
<tr>
<td>0.25</td>
<td>298 223 260</td>
<td>189 175 182</td>
</tr>
<tr>
<td>5</td>
<td>398 237 317</td>
<td>209 122 165</td>
</tr>
<tr>
<td>10</td>
<td>254 96 175</td>
<td>184 133 158</td>
</tr>
<tr>
<td>15</td>
<td>118 80 99</td>
<td>151 158 154</td>
</tr>
<tr>
<td>20</td>
<td>300 190 245</td>
<td>285 125 235</td>
</tr>
</tbody>
</table>

1. Trial number
2. CPM is 5 ul samples containing 2 x 10^6 washed erythrocytes drawn from cultures containing 1 x 10^9 erythrocytes and 20 uCi $^3$H-thymidine or $^3$H-uridine per ml.
3. Cultures contained blood diluted 1:6 in Temin's Modified MEM. The parasitemias of infected populations of erythrocytes were about 20%. Leukocytes were removed by filtration through filter paper powder columns. Each culture contained about 2 ml with 1 x 10^9 erythrocytes per ml.
Table 2. Dynamics of $^3$H-glucosamine incorporation by *P. berghei* infected and uninfected erythrocytes.

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Erythrocytes</th>
<th>Culture Supernatants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected 1</td>
<td>Infected 2</td>
</tr>
<tr>
<td>9.25</td>
<td>44</td>
<td>118</td>
</tr>
<tr>
<td>3.00</td>
<td>196</td>
<td>183</td>
</tr>
<tr>
<td>5.00</td>
<td>613</td>
<td>538</td>
</tr>
</tbody>
</table>
Table 3. Dynamics of incorporation of $^{35}$S-methionine by *P. berghei* infected or uninfected erythrocytes.

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Infected Reticulocyte-Rich (20% Infected Cells)</th>
<th>Infected Mature RBC's (2% Infected Cells)</th>
<th>Uninfected Reticulocyte-Rich</th>
<th>Uninfected Mature RBC's</th>
<th>Culture Fluid In- uninfected Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>Ave.</td>
<td>1</td>
</tr>
<tr>
<td>0.25</td>
<td>2211</td>
<td>1010</td>
<td>1611</td>
<td>.....</td>
<td>218</td>
</tr>
<tr>
<td>5</td>
<td>22954</td>
<td>3747</td>
<td>4861</td>
<td>12521</td>
<td>447</td>
</tr>
<tr>
<td>10</td>
<td>17670</td>
<td>6379</td>
<td>7232</td>
<td>10457</td>
<td>1345</td>
</tr>
<tr>
<td>15</td>
<td>30301</td>
<td>8684</td>
<td>9127</td>
<td>16037</td>
<td>1663</td>
</tr>
<tr>
<td>20</td>
<td>32111</td>
<td>5954</td>
<td>7099</td>
<td>15055</td>
<td>1388</td>
</tr>
</tbody>
</table>

1. Cultures contained blood diluted 1:6 in Temin's Modified MEM. Leukocytes were removed by filtration through a filter paper column. Each culture contained 2 ml with $1 \times 10^9$ cells per ml.

2. Trial number

3. CPM in 5 ul samples containing $2 \times 10^6$ washed erythrocytes drawn from cultures containing $1 \times 10^9$ erythrocytes and 20 uCi $^{35}$S-methionine per ml.
Table 4. Dynamics of $^{35}$S-methionine incorporation by *P. berghei* infected and uninfected erythrocytes labelled in 5 hour pulses.

| Pulse time | Infected Reticulocytes | | | Uninfected Reticulocytes | | |
|------------|------------------------|-----------|-----|------------------------|-----------|
|            | Trial 1 | Trial 2 | Ave. | Trial 1 | Trial 2 | Ave. |
| 0 - 5      | 8961    | 7293    | 8127 | 616     | 1007    | 811  |
| 5 - 10     | 1751    | 1756    | 1753 | 334     | 337     | 335  |
| 10 - 15    | 469     | 225     | 347  | 471     | 294     | 382  |
| 15 - 20    | 258     | 208     | 233  | 217     | 393     | 255  |

1. Cultures contained blood diluted 1:6 in Temin's Modified MEM. Leukocytes were removed by filtration through filter paper columns. Each culture contained about 2 ml of fluid and 1 x 10^6 cells per ml.

2. CPM in 5 ml samples containing 2 x 10^6 erythrocytes drawn from cultures containing 1 x 10^6 erythrocytes and 20 uCi $^{35}$S-methionine per ml.
Table 5. Dynamics of $^{35}$S-methionine incorporation by *P. falciparum* infected and uninfected human erythrocytes labelled in 12 hour pulses. RPMI 1640 or Temin's Modified Minimal Essential Medium

<table>
<thead>
<tr>
<th>Trial #</th>
<th>Infected Erythrocytes</th>
<th>Uninfected Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RPMI</td>
<td>MEM</td>
</tr>
<tr>
<td>1</td>
<td>1023</td>
<td>845</td>
</tr>
<tr>
<td>2</td>
<td>695</td>
<td>600</td>
</tr>
<tr>
<td>3</td>
<td>756</td>
<td>618</td>
</tr>
<tr>
<td>4</td>
<td>639</td>
<td>400</td>
</tr>
<tr>
<td>average</td>
<td>778</td>
<td>615</td>
</tr>
</tbody>
</table>
Table 6. Relative mobilities of standards used for the determination of molecular weights.

<table>
<thead>
<tr>
<th>STANDARD</th>
<th>MOLECULAR WEIGHT</th>
<th>7.5% acryl.</th>
<th>10% acryl.</th>
</tr>
</thead>
<tbody>
<tr>
<td>soybean trypsin</td>
<td>21,500</td>
<td>1.0</td>
<td>0.82</td>
</tr>
<tr>
<td>inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carbonic anhydrase</td>
<td>31,000</td>
<td>0.96</td>
<td>0.58</td>
</tr>
<tr>
<td>ovalbumin</td>
<td>45,000</td>
<td>0.78</td>
<td>0.39</td>
</tr>
<tr>
<td>BSA</td>
<td>66,200</td>
<td>0.52</td>
<td>0.20</td>
</tr>
<tr>
<td>phosphorylase B</td>
<td>92,500</td>
<td>0.37</td>
<td>0.12</td>
</tr>
<tr>
<td>galactosidase</td>
<td>116,250</td>
<td>0.22</td>
<td>0.08</td>
</tr>
<tr>
<td>myosin</td>
<td>200,000</td>
<td>0.10</td>
<td>0.02</td>
</tr>
</tbody>
</table>
APPENDIX II: Determination of Molecular Weights
Figure 27. Standard Curve used for Determination of Molecular Weights.
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