Bulletin of the World Health Organization, 55 (2-3): 317-331 (1977)

The isolation and fractionation of malaria-infected cells *

JULIUS P. KREIER [1]

This paper is a critical review of procedures for the isolation of malarial parasites from host cells and their fractionation. The procedures are grouped according to the stage of parasite being isolated, and the procedures for isolation of the erythrocytic stages are further grouped by techniques used. Some types of procedure are described for isolation of all stages of the parasite, both those in the invertebrate and vertebrate hosts. The uses and limitations of the various procedures are described. It is concluded that all the procedures are useful for some purposes, but that from a morphological standpoint only natural release in culture and continuous flow oscillation provide large yields of intact erythrocytic parasites free of host cell membranes.

The malarial parasite is a complex eukaryotic organism. The parasite develops in two hosts, an invertebrate and a vertebrate, and in those hosts it exists in a variety of morphologically distinct forms. In each host a variety of organ and cell systems are parasitized. Some of the stages exist only intracellularly, whereas others such as sporozoites and merozoites are specialized for passage from one host, or host cell, to another. The morphologist describes the organism in terms of plasma membranes, pellicule complexes, microtubules, nuclei, ribosomes, and a multitude of other structures; the biochemist talks about proteins, lipids, and carbohydrates; the physiologist discusses enzymes, and the immunologists and serologists are concerned with antigens. They are all, of course, talking about the components of the same parasite; only their terminology is different and reflects their training and interests. They are all confronted with the same problem, that of obtaining sufficient quantities of the parasite in forms suitable for their purposes. The problem of obtaining sufficient quantities of the parasite for study is complicated by the fact that the parasite has a complex life cycle. While all stages of the parasite almost certainly share common components (*79*), each stage also has unique components characteristic of the stage. Thus, even if it were possible to eliminate the problems in analysis caused by the parasite's intimate association with its host, it still remains necessary to separate the parasites by stage of development, and to separate the components of each stage of the parasite to determine which are common to all stages and which are unique. As some stages of the parasite may undergo antigenic variation (*17*), another level of complication is added to the analysis of the components of the parasite.

In this review I will attempt to summarize the literature which describes attempts by various people to isolate malaria parasites and to fractionate them into their component parts for study. A number of reviews and reports are available that contain sections on isolation and fractionation of malaria parasites and provide information on the implications of isolation and fractionation work (*164*, *161*, *13*, *107*, *162*, *89*, *49*, *50*, *155*, *1*).[a]

ISOLATION AND FRACTIONATION OF STAGES OF THE PARASITE FROM THE VERTEBRATE HOST

Exoerythrocytic stages including exoerythrocytic merozoites

The mass *in vitro* cultivation of exoerythrocytic stages of avian malaria parasites in cell monolayers

* This work was supported in part by a grant (DADA 17-72-2072) from the US Army Medical Research and Developments Command and is paper No. 1457 from the Army Malaria Project.

[1] Professor, Department of Microbiology, Ohio State University, Columbus, 43210, USA.

[a] See also National Academy of Sciences. *Workshop on problems related to the development of an antimalarial vaccine*. National Research Council, Washington, DC, 1974 (mimeographed document).

is technically feasible (*39*, *68*). Exoerythrocytic merozoites may be obtained by centrifugation of the cell culture medium overlay at 4000 *g* for 30 min (*64*). Immunization of turkeys with formalin-killed exoerythrocytic merozoites prepared from such cultures conferred some stage-specific protection against exoerythrocytic forms of the parasite, but did not protect against infection by blood forms (*64*). Some evidence exists that injection of the avian merozoites will confer protection against infection with *P. berghei* sporozoites (*65*).

Plasmodium berghei yoelii exoerythrocytic schizonts stain well with fluorescent-labelled serum raised to blood stages of the parasite (*48*), indicating either that the fluorescent antibody test was not measuring response to antigens important in stimulating immunity or that, in rodent malaria, antigens stimulating protection are shared by exoerythrocytic and erythrocytic stages. The *in vitro* cultivation of exoerythrocytic stages of mammalian plasmodia, a prerequisite for studies of the antigenic constituents of this stage of the parasite, is very difficult. This difficulty arises partly from the fact that exoerythrocytic stages of mammalian plasmodia, unlike those of avian plasmodia, undergo only a limited number of generations of reproduction and must, therefore, always be produced from sporozoites, and partly because their host cell, the liver parenchyma cell, is difficult to cultivate. It is difficult to obtain sterile sporozoites from conventionally reared mosquitos, and this also complicates the culture procedure (*67*). Avian exoerythrocytic parasites will grow readily in mammalian liver cells (*8*), but only sparse growth of mammalian parasites in such cells has been reported (*44*).

Erythrocytic stages

General considerations. There is a very extensive literature on procedures for obtaining plasmodia from infected red cells. By some approaches, the infected red cells and the contained parasites are disrupted and then various biochemical or biophysical techniques are used to obtain the desired parasite components from the soluble mixture; by other approaches, an attempt is made to free morphologically intact parasites from the red cells and then to separate these parasites from the erythrocyte debris before solubilization and fractionation of the parasites is undertaken. Practically all of the procedures used for obtaining plasmodial components from red cells are variations of a few distinct techniques; these are hypotonic lysis, lysis by freezing and thawing, lysis with agents such as saponin, NH_4Cl, or antiserum and complement, lysis by sudden decreases in pressure, lysis by ultrasound, and the most recent procedure, cultivation to permit the parasites to mature and be released spontaneously. The first two of these procedures provide a soluble mixture of parasite and host components which must then be fractionated; the remainder of the procedures, with varying degrees of success, provide parasites freed from the host cells for further study.

Preparation of parasitized erythrocytes. The most economical and convenient source of erythrocytes infected with plasmodia for laboratory study is the blood of rodents infected with one of the various rodent plasmodia. The skills and knowledge needed for isolation and fractionation of plasmodia can easily be obtained using rodent material. Avian plasmodia are less suitable for study of isolation and fractionation of plasmodia, as the presence of the erythrocyte nucleus and its contained DNA complicate separation procedures. Parasitized blood cells can also be obtained from monkeys infected with their respective malarias if desired (*24*). The demonstration that *Plasmodium vivax* (*99*) and *Plasmodium falciparum* (*54*) grow well in *Aotus trivirgatus*, and the more recent successful *in vitro* culture of *Plasmodium falciparum* in erythrocytes (*147*, *62*), provide sources, other than infected humans and chimpanzees, of erythrocytes containing human plasmodia for study. Culture of erythrocytic stages of plasmodia in cells other than erythrocytes (*132*) is not yet a practical procedure.

Blood from animals or humans infected with plasmodia may be subjected to some preliminary treatments to remove unwanted components and to increase the proportion of infected erythrocytes before separation of the parasites from the host cells is undertaken. A first step is washing in physiological saline to remove plasma. If, during the washing steps, the buffy coat is removed along with the diluted plasma, the numbers of platelets and leucocytes will also be reduced. A more complete removal of leucocytes can be accomplished by passage of the resuspended, washed blood cells through a column of filter paper powder (*53*). Blood diluted 1: 6 in an appropriate medium may be passed directly through a column of filter paper powder (*104*). The column of powder should be wet with saline solution before use. The ability of the columns to retain leucocytes is limited. They can be overloaded, and then the

excess leucocytes will pass through. Forcing passage by pressure, or by excessive rinsing, to increase the speed of the process or to decrease the loss of red cells will dislodge leucocytes from the column. Attempts have also been made to remove leucocytes from infected blood by gradient techniques. The range of densities of leucocytes (*160*) overlaps that of parasitized red cells and thus these procedures are not very good. Of the various procedures reported for the removal of leucocytes from infected blood, the filter paper powder column technique, when used properly, is the best (*66*). Platelets may be removed by passage of the blood through a glass bead column (*114*).

Before one attempts to separate the parasite from the host red cell, it may be desirable to separate the parasitized red cells from the uninfected ones, or to attempt the separation of parasitized red cells by stage of development. Plasmodia are less dense than red cells. The more parasites a red cell contains, and the larger the parasites it contains, the less dense the host cell–parasite complex. Some plasmodia cause the red cell to enlarge, decreasing its density, and some plasmodia preferentially infect young erythrocytes which are less dense than mature erythrocytes. All these factors affect the isolation of parasitized erythrocytes by gradient techniques (*93*).

Eaton (*46*) collected schizont-containing rhesus monkey erythrocytes by allowing infected blood to settle slowly. The schizont-containing cells were present in increased concentration, just under the buffy coat. Gradients for separation may be made from sucrose (*156*), human albumin (*52*), bovine albumin (*111*), or phthalate (*93*). In very recent studies, Lund & Powers (*82*) concentrated schizont-containing *P. knowlesi*-infected erythrocytes on a discontinuous Ficoll gradient. Many schizont-containing cells were in a layer at the interface of the 20 and 25% Ficoll bands. McAlister & Gordon (*88*) separated *P. berghei*-infected cells by stage of development on a discontinuous Stracten II gradient. Schizont-containing cells had a specific gravity of less than 1.043, trophozoite-containing ones between 1.081 and 1.091, and uninfected erythrocytes and erythrocytes containing very small parasites had specific gravities greater than 1.091. Sucrose is an undesirable substance for gradient preparation because it is osmotically active. All reported studies have been done with parasitized erythrocytes, none with free parasites, although some of the workers do not make the distinction in their discussions.

For those working with a synchronized infection, parasites primarily in a given stage of development may be obtained by judicious choice of the time of blood collection. This system was used by Trager et al. (*148*) to obtain a population of *P. lophurae* trophozoites. If, however, the parasites are, as *P. berghei*, not naturally synchronized or lose their synchrony in culture, then one must either use techniques such as the gradient centrifugation procedures just described to obtain parasites in a given stage, or attempt to induce synchrony. Arnold and his associates (*5*, *125*) demonstrated that some degree of synchronization of *P. berghei* in mice could be induced by control of the photoperiod to which the host mice were exposed. Successful synchronization of *P. berghei* infection would facilitate isolation of specific stages of *P. berghei* from the blood, and greatly simplify the analysis of the developmental events in this otherwise most easily studied malaria parasite.

Hypotonic lysis. Some of the earliest successful attempts to prepare plasmodial antigens for serodiagnosis used hypotonic lysis of parasitized erythrocytes to release antigens. Coggeshall & Eaton (*25*) prepared a group-specific complement-fixing antigen from *P. knowlesi*-infected rhesus monkey erythrocytes by treating infected erythrocytes, probably at 37°C, for 48 hours with three times their volume of distilled water. After centrifugation, the haemoglobin-containing supernatant fluid was made isotonic by addition of NaCl. Later, the procedure was modified (*45*). The haemoglobin-containing fluid, produced by the distilled water lysis, was discarded and the solid material, containing a mixture of erythrocytic stroma and parasites in various stages of disruption, was collected. This material was then dried under vacuum and stored until needed. When it was to be used, it was ground, saline solution added, and then freeze–thawed three times. After centrifugation, the clear supernatant fluid was used for antigen in complement fixation tests. The material had the same type of group-specificity as that in the lysate used by Coggeshall & Eaton (*25*), but had the advantage of being relatively free of haemoglobin. Davis (*39*) separated the antigen from the haemoglobin in the crude lysate by precipitation of the antigen from the lysate. He did this by adding 35 g of $(NH_4)_2SO_4$ to each 100 ml of lysate.

Antigens prepared by procedures very similar to those of Dulaney & Stratman-Thomas (*45*) have been used by Mayer & Heidelberger (*86*) in studies

of the CF test in the diagnosis of malaria; by Stein & Desowitz (*138*) to sensitize red cells for use in a passive haemagglutination test; and by Chavin (*21*) as starting material for an analysis of parasite constituents.

Lysis by freezing and thawing. The freezing and thawing of the infected erythrocytes causes haemolysis and also damages the parasite's membrane. Following centrifugation to remove the membraneous structures, one obtains a deep red fluid which contains, in addition to haemoglobin, a variety of soluble products from the parasite and the host cell. The soluble parasite products obtained are commonly used in serodiagnostic tests. In the procedure used by Sadun & Gore (*112*), the infected cells are washed to remove the plasma, then a 50% suspension of the washed cells is prepared in saline and subjected to three cycles of freezing and thawing. The crude haemolysate is centrifuged at 2700 *g* for 30 min to remove insoluble components, and then may be stored frozen at −70°C until used. Sadun & Gore (*112*) fractionated the lysate by sequential elution from a Sephadex A-25 column with phosphate buffers of increasing concentrations. Haemoglobin was dislodged from the column by a 0.04 mol/litre buffer, pH 7.5, while a parasite fraction that was dislodged by a 0.1 mol/litre buffer, pH 6.5, proved to be excellent as a diagnostic antigen in the soluble antigen fluorescent antibody test. No attempt was made to determine what the antigen was, but Sadun & Gore (*112*) suggested that lysis of the host cell–parasite complex should conserve soluble parasite products that would be lost by procedures which lysed the erythrocyte to remove haemoglobin before the parasite was extracted. McAlister (*87*) examined the insoluble material left after freeze–thawing by a procedure similar to that of Sadun & Gore (*112*). He found some unlysed erythrocytes, many morphologically intact parasites, particularly trophozoites, and much stromal material. McAlister (*87*) found that the serologically active materials could be precipitated from the haemoglobin-containing solution by adding $(NH_4)_2SO_4$, and that the precipitated material, after solution, could be further purified by passage through a G-100 Sephadex column. The antigenically active material obtained from the column was in the void volume and had a molecular weight of at least 800 000. McAlister (*87*) confirmed that the antigen was useful in a soluble antigen fluorescent antibody test and also showed its usefulness in a passive haemagglutination test. Mathews et al. (*85*) used antigen released by freeze–thawing in passive haemagglutination tests for malaria. The antigen they routinely use for sensitizing the carrier erythrocytes is a crude lysate of parasitized erythrocytes obtained by freezing and then thawing the washed infected erythrocytes, passing the crude lysate through a Ribe Cell Fractionator at a pressure of 117.2 MPa, and then centrifuging the lysate at 12 000 *g* for 10 min. The selective absorption of the serologically active antigens on to the tanned red cells is the only additional fractionation used. A variant of this type of procedure subjects the freeze-thawed parasitized erythrocytes to a 10-second burst of sonic energy, instead of passing it through a Ribe Fractionator before it is used to sensitize the red cells (*91*). Unfractionated freeze–thawed *P. knowlesi* schizont-containing erythrocytes have been used as antigen in vaccination studies (*18*). The materials appear to stimulate a moderate degree of immunity to challenge infection.

Lysis with saponin. The use of saponin to lyse erythrocytes, and centrifugation to collect the unlysed parasites, was introduced by Christopher & Fulton (*22*). The procedure, which has been changed little by subsequent workers, is widely used today as the first step in reducing the proportion of host material in parasite preparations. Stauber & Walker (*136*) recognized that parasites prepared by saponin lysis of parasitized erythrocytes were, with rare exceptions, contained within the collapsed erythrocyte membranes. They recognized this because the avian parasites with which they worked remained bound to the host cell nucleus after lysis of the erythrocyte. The erythrocyte membranes surrounding the parasites cannot be resolved by light microscopy of Giemsa-stained films of the parasites, and it is owing to this fact that the mistaken view that saponin lysis yields free parasites arose. Phase-contrast microscopy of wet preparations, or electron microscopic examination of thin sections of the parasites (*2*, *28*, *100*, *101*, *102*), is required to demonstrate the erythrocyte membrane envelope around the parasites.

Dr A. Zuckerman has been a major force in the reintroduction and popularization of the saponin lysis technique (*134*) and her adaptation of the original procedure has been described in detail (*165*). In brief, Zuckerman's procedure is as follows. The blood is collected in a suitable anticoagulant solution, for example, sodium citrate. The plasma is removed following centrifugation and the erythrocytes are washed in phosphate-buffered saline by a

series of centrifugations. Leucocytes and platelets may be removed in part with the plasma if the buffy coat is also removed. The blood cells suspended in buffered saline may be further purified of leucocytes and platelets by sedimentation through a 3.6% dextran solution. The erythrocytes clump and sediment rapidly; the leucocytes are discarded with the supernatant fluid.

The sedimented cells are washed in phosphate-buffered saline and then 5 times their volume of a solution that contains 1 part of saponin in 7500 parts of saline at 37°C is added. The mixture is incubated at 37°C for 15 min with occasional stirring. The suspension is then chilled and centrifuged for 30 seconds at 10 000 rev/min. The supernatant fluid is discarded and the procedure repeated, the only difference being that incubation in saponin is only for 10 min, not 15. The parasites and erythrocyte membranes are in the pellet after the last centrifugation, and are ready for use after washing in saline to reduce the saponin contamination.

Dr Zuckerman recognized the importance of eliminating leucocytes from the parasitized erythrocytes before lysis; however, the dextran procedure she recommends, as mentioned earlier (page 319), is not as good as the filter paper column technique for removing leucocytes.

Since it has been recognized that the parasites prepared by saponin lysis are contained in the erythrocyte membrane, attempts have been made to remove the membrane. Stauber & Walker (*136*) used enzymatic digestion. The digested parasite preparations, unlike the undigested preparations, could be agglutinated by immune serum, but were not free of membranes (*149*). Kreier et al. (*77*) fixed saponin-prepared avian parasites with formalin and then separated the parasites from the erythrocyte nuclei by sonication. These free parasites were morphologically intact and could be agglutinated with immune serum but were not suitable for solubilization and further analysis because of the fixation. Cook et al. (*28*) attempted to remove the erythrocyte membranes from *P. knowlesi* prepared by saponin lysis by a variety of techniques. They observed that fixation with glutaraldehyde followed by mechanical agitation in a Waring blender would yield morphologically intact, free parasites, but they observed that these fixed parasites were of little use for subsequent studies. They also found that mechanical agitation without prior fixation disrupted the parasites, and that treatment with sodium dodecyl sulfate lysed the parasites.

Most scientists using the saponin-prepared parasites have accepted the presence of the erythrocyte membranes in the preparations, and have tried to evaluate their importance by comparative study of saponin-prepared erythrocyte membranes.

For most studies of the parasites, the collection of the morphologically intact parasites after lysis of the host erythrocytes with saponin, is only the first step in the process. The second step is generally the disruption of the collected parasites and the separation of the soluble from the insoluble material by centrifugation. A great variety of procedures have been used to disrupt the parasites. Sherman & Hull (*124*) obtained proteins and haemozoin from *P. lophurae* for biochemical analysis by alternate freezing and thawing, and Sherman (*123*) used freeze–thawed parasites for antigenic analysis. Cook et al. (*29*) obtained ribosomes from *P. knowlesi* after disruption of the saponin-prepared parasites by distilled water lysis. Zuckerman introduced the use of the Hughes press (*134*) to disrupt the parasites and carried out analysis of the soluble components by disc gel immunodiffusion and immunization techniques (*135*, *60*, *61*). A variety of techniques have been used for grinding the parasites. Jerusalem (*70*, *72*) used a Potter Homogenizer to grind the parasites. Corradetti et al. (*30*) first filtered the saponin-lysed material, then ground the parasites obtained in the filtrate in a Virtis blender with quartz powder, and Rock et al. (*105*) ground the parasites in an all-glass Ten Broeck homogenizer.

A variety of individuals have used ultrasound either alone or with other procedures to disrupt the parasites (*43*, *131*, *153*, *83*). In some cases the parasites were lyophilized before grinding (*12*, *159*).

Lysis with ammonium chloride. Plasmodia may be freed from erythrocytes by treatment of the infected erythrocytes with a 0.155 mol/litre NH_4Cl solution buffered to pH 7.4 with 0.17 mol/litre Tris buffer. One volume of a 50% erythrocyte suspension is added to 9 volumes of the 37°C buffered NH_4Cl solution and the mixture incubated at 37°C for 3 min. Subsequently, the mixture is centrifuged at 500 *g* for 12 min at room temperature and the sediment is washed twice in a MEM solution with 10% fetal calf serum. The parasites in the sediment are reported to be free of red cell membrane, morphologically intact, and suitable for antigenic studies (*84*). The crucial importance of controlling the time of exposure of the parasites to the lytic solution, if the parasites are not also to be lysed,

was emphasized in a later study of the technique (*103*).

Lysis with antiserum and complement. The release of plasmodia from host erythrocytes by use of specific anti-host erythrocyte antiserum and complement has been used most commonly by individuals culturing free parasites (*145*, *146*), and by individuals studying plasmodial metabolism and enzyme activity (*11*, *80*, *150*). A fairly typical procedure is that of Trager et al. (*148*). Washed infected erythrocytes are suspended in four times their volume of a balanced saline solution containing a lysed erythrocyte extract. To 6.3 ml of the suspension, 0.13 ml of guinea-pig serum and 0.7 ml of rabbit origin anti-erythrocyte serum are added. The mixture is incubated for 30 min at 40°C on a rocking platform, and is swirled vigorously after 15 min and again at the end of the 30-min incubation period. The material is then sucked in and out of a pipette and transferred to a centrifuge tube and centrifuged just long enough to result in clearing of the fluid. The freed parasites are in the supernatant. Earlier versions of the procedure (*145*) included enzyme digestions with trypsin and deoxyribonuclease, but these preparations had lowered viability (*148*). Some procedures passed the supernatants obtained from the centrifuged lysate through a 5-μm filter at a pressure of 82.7 kPa (*15*).

Langreth & Trager (*81*) presented electronmicroscopic evidence that *P. lophurae* trophozoites prepared by immune lysis were free of host membranes, and sufficiently undamaged that they would develop into schizonts extracellularly in culture. An interesting variant of the immune lysis procedure was that of Walter (*152*), who brought about *in vivo* release of *P. berghei* parasites by immunizing mice with normal rat erythrocytes and then infecting the mice with *P. berghei* parasitized rat erythrocytes. As the resulting infections were synchronized for several generations, Walter (*152*) concluded that only the merozoites released by the *in vivo* haemolysis were infective. This method of achieving synchrony, if repeatable, would facilitate evaluation of the components of the intraerythrocytic development stages of the normally unsynchronized *P. berghei*.

Lysis by sudden decreases in pressure. The technique for release of plasmodia from erythrocytes by decompression under the controlled conditions given by a French pressure cell, was developed by D'Antonio (*37*). A 20% suspension of washed infected erythrocytes is prepared in a buffered saline solution. This is extruded from the orifice of the French pressure cell at an appropriate pressure, usually between 5.5 and 6.9 MPa. The pressure chosen depends on the parasite–host cell system being studied, and must be selected on the basis of trial and error (*34*). The effluent from the cell is centrifuged at 50 *g* for 10 min to remove the unbroken red cells and gross debris, and then the parasites are collected by centrifugation of the supernatant at 3500 *g* for 5 min. The parasites are washed, then suspended in 7 volumes of saline and disrupted by passage through the French pressure cell at 124–138 MPa. The effluent is cleared by centrifugation at 10 000 *g* for 30 min (*37*). The supernatant fluid from this centrifugation was used in a complement fixation test (*38*). On passage through a G-200 Sephadex gel column the material in the void volume proved to contain most of the CF activity. The CF antigen is stable for 2 months at 4°C and may be lyophilized without loss of activity if 1% polyvinylpyrrolidone is added (*37*).

Parasites prepared by the French pressure cell technique have been used to study the enzymes of *P. knowlesi* (*114*). On fractionation these parasites have yielded a lytic factor of lipid nature which lyses normal erythrocytes (*51*). The most extensive use of French pressure cell released plasmodia and their fractions has been in immunization experiments in Dr Silverman's laboratory (*35*, *36*, *127*, *115*, *116*). Speer et al. (*133*) reported that the immunizing material in the void volume of G-200 Sephadex column fractionated, French pressure cell released, and disrupted plasmodia was largely vesicles of parasite membrane origin.

Chow & Kreier (*23*) obtained free parasites by the very simple technique of extruding a 10% suspension of washed *P. berghei*-infected red cells through a 27-gauge needle by hand pressure on the syringe plunger. The approximate rate of extrusion was 0.5 ml/s. The effluent was diluted four-fold, and then centrifuged briefly at low gravity force to remove unbroken red cells; the supernatant was then centrifuged at 3500 *g* for 8 min to collect the parasites. This very simple procedure, within the reach of almost any laboratory, yielded parasites suitable for studies of the action of phagocytes on free parasites and parasitized erythrocytes.

Lysis by ultrasound. It was shown in 1956 (*151*), that ultrasound would disrupt red cells and release plasmodia. Later, it was shown that parasite yield increased only to a limited degree with increases in the intensity of the sonic energy used, and the

duration of the exposure (*108*), and that while low frequency ultrasound waves appeared to disrupt red cells more efficiently than high frequency waves, no frequency existed that would break red cells but not plasmodia (*102*). It is thus apparent that in batch sonication systems, no combination of time, intensity, and frequency can be selected that will break red cells without also subsequently breaking the released parasites. Thus, the problem in the use of sonic energy for release of plasmodia was one of developing a system to permit red cell disruption, and then achieve prompt removal of the freed parasites from the sound field. This problem was resolved by the development of a continuous flow system (*100*). The crucial technical aspect of the system is the design of the chamber. It must have a small void volume. The washed parasitized red cells must pass through the ultrasound field in an ordered fashion, and there must be no eddies in which parasitized erythrocytes and parasites may be trapped and subjected to prolonged exposure to the disruptive forces of the ultrasound. The optimum rate of flow for maximum yield of parasites must be selected by experimentation for each continuous flow system. Almost any reasonably powerful commercial sonicator may be used if fitted with a chamber that permits the ordered passage of a thin layer of suspended parasitized cells through the sound field. A satisfactory system has been described in detail (*100*). Leucocytes and platelets must be removed from the blood cells by a suitable procedure before sonication (page 318), for sonication will cause release of nucleic acids which entrap the parasites and prevent subsequent separation of the parasites from the erythrocyte debris. Prior & Kreier (*100, 101*) separated the freed parasites from unbroken erythrocytes and erythrocyte debris by differential centrifugation. A short, low-force centrifugation is used to remove the unbroken red cells, then a longer, higher-force centrifugation pellets the parasites, and the finely divided debris remains in the supernatant fluid. A practical procedure to select the conditions of centrifugation required to obtain parasites is to prepare a series of identical tubes of the effluent from the sonication chamber, then choose an arbitrary gravity force, for example 300 *g*, and centrifuge for 5, 10, 15, 20, 25, and 30 minutes. Examination of the pellets will permit choice of appropriate centrifugation times at the selected gravity force to obtain a clean parasite preparation. Higher gravity forces may be used with shorter times. Alternatively, the supernatant fluid from a single sample may be centrifuged several times at a constant gravity force and each pellet examined to determine the appropriate times for centrifugation to obtain clean parasites.

Thin-section electron micrographs have been published showing that relatively undamaged parasites, free of entrapping erythrocyte membranes, may be prepared by the sonication technique (*100, 101, 76*). Parasites prepared by continuous-flow sonication have been shown to be suitable as sources of antigen for complement fixation tests (*100, 101*), for studies of the mode of action of the host against the parasite (*57, 58, 59*), and in vaccination studies (*113*). The excellent state of preservation of the sonically freed parasites has made them very useful for the study of the surface properties of free parasites, an area not previously amenable to study (*117, 119, 121*). The technique is not limited to use with plasmodia; *Babesia* have been freed by essentially the same procedures (*55, 75*).

Natural release. All of the procedures for obtaining erythrocytic stages of plasmodia described so far in this review have involved the use of chemical or physical forces to disrupt the host erythrocyte membrane to release the contained parasites, or to release components of the parasites. The parasites obtained are in whatever stage of development they were in at the time of erythrocyte rupture. Mitchell et al. (*94*) were able to culture erythrocytes containing *P. knowlesi* schizonts long enough for these schizonts to mature and release merozoites which were then collected by differential centrifugation. Later, Dennis et al. (*41*) improved the procedure by culturing the schizonts in a chamber, the floor of which was a 0.2-μm pore size polycarbonate sieve through which the merozoites were drawn as they were released. More recently, *in vitro* techniques for short-term culture (*126*) and finally serial culture of *P. falciparum* (*147, 62*) have been developed. Culture of *P. falciparum* in a chamber like that used by Dennis et al. (*41*) with *P. knowlesi* will permit the collection of *P. falciparum* merozoites in quantity. Culture techniques for other plasmodia, *P. berghei* (*129*) for example, are less advanced. Despite the newness of the natural release techniques for obtaining erythrocytic merozoites, the usefulness of the culture procedures for obtaining merozoites for study of host–parasite interactions (*26, 92*) is proved. The most significant use of the merozoites obtained by natural release has been in vaccination studies (*32, 95, 96*).

Comparison of procedures. When one is evaluating procedures for separating plasmodia from host cells, or for obtaining plasmodial components, one must realize that the value of the procedures is to a large degree determined by the objectives of the individual making the study. In general, the simplest procedure that provides parasite components in a form satisfactory for the studies being done is the best procedure. Thus Kagan and his associates have found an admittedly crude lysate of infected red cells satisfactory for sensitizing tanned red cells for use in seriodiagnosis (*85*), and Sadun & Gore (*112*) used a freeze–thawed lysate of whole infected red cells as the starting material for fractionation to obtain an antigen for a soluble antigen fluorescent antibody test. These workers considered that the materials present in the red cell cytoplasm would be lost by preliminary freeing of the parasites from the host cells. Lund & Powers (*82*) found that for a passive haemagglutination test the antigen obtained by freeze–thawing schizont-infected red cells was as good as the antigen obtained from saponin-prepared parasites, but the yield from the free parasites was smaller, indicating that antigen may be lost with the haemoglobin as suggested by Sadun & Gore (*112*). D'Antonio et al. (*37*) compared the value, in diagnostic CF tests, of antigens prepared by French pressure cell lysis of infected erythrocytes to ones prepared by the older distilled water lysis techniques. The French pressure cell antigens were less anticomplementary and more potent than were those prepared by distilled water lysis. Prior & Kreier (*101*) found that sonically freed parasites provided excellent CF antigens, but they did not make direct comparisons with other antigens. No systematic evaluation of plasmodial antigens for use in serology appears to have been undertaken. The techniques for systematic evaluation of antigens for specificity and potency have been described in detail (*49*, *50*) and need only be applied systematically.

Comparative evaluation of various antigen preparations for immunization is an undeveloped field. Jerusalem & Eling (*71*) made a comparison of the immunogenicity of saponin-released, haemolytic antiserum and complement-released, and dilute formalin-released *P. berghei* as immunogens in mice. They concluded that the method of release of the parasite was not related to the antigenicity. Desowitz (*42*) compared the immunogenicity of several subfractions of saponin-prepared *P. berghei* parasites in rats in what was basically a study of adjuvants. He reported that alum-precipitated components of the soluble portions of saponin-prepared *P. berghei* parasites were not immunogenic. Saul & Kreier (*113*) compared the immunogenicity of various fractions of sonically-lysed *P. berghei* in rats. The techniques they describe could be applied to a comparison of the various antigens currently being used. These workers emphasize the importance of a careful quantitative analysis of the immunogens if valid comparisons are to be made. Most proposed vaccines have not been tested against other proposed vaccines in any systematic way.

Since many claims have been made that the procedures for preparing plasmodia would yield morphologically intact parasites free of host membranes, it was perhaps inevitable that electron microscope techniques would be used to evaluate the claims. Bahr (*6*) concluded that no procedure available at the time he made his study yielded morphologically intact parasites free of host cell membranes. Kilby & Silverman (*73*) made a fairly careful comparison of *P. berghei* released by the French pressure cell, saponin, antiserum and complement, and distilled water techniques and they concluded that lysis by distilled water disrupted the parasites but did not remove host membranes; saponin yielded parasites still in host membranes; and antiserum and complement did not free many parasites. They reported that the French pressure technique released the largest proportion of structurally intact parasites generally lacking closely associated host cell membranes. Cook et al. (*28*) and Cook & Aikawa (*27*) disputed Kilby & Silvermans' conclusions about the French pressure cell technique, as did Trager et al. (*148*). These individuals provided sound evidence that the French pressure cell procedure frees few parasites from host membranes, and disrupts many of those that are freed. Cook et al. (*28*) considered that the saponin procedure provided the largest yield of morphologically intact parasites, but observed that these are in host cell membrane ghosts. Aikawa & Cook (*2*) confirmed that saponin does not remove host cell membrane from around parasites and that the French pressure cell produces a disrupted mixture of materials. Aikawa & Cook (*2*) emphasized that morphological evaluation requires low power electron microscope scans of fields of parasites to be of any value. They noted that all procedures will provide some free parasites.

The two procedures that actually provide good

yields of morphologically intact free erythrocytic parasites, i.e., continuous-flow sonic oscillation and natural release in culture, did not exist at the time the comparisons just discussed were made. Adequate electron microscopic evidence of the efficacy of these two procedures for freeing parasites exists (*100, 101, 103, 76, 41*). The natural-release procedure yields merozoites; the sonic procedure yields free parasites of normally intraerythrocytic types.

The identification of erythrocyte membrane material in disrupted parasite preparations has been a problem. Erythrocyte membranes will fix complement with sera of individuals with malaria (*63*), and the active components, which are at least in part lipids, will sensitize erythrocytes for haemagglutination and will precipitate in gel with serum from infected individuals (*120*). It has recently been shown that erythrocyte membranes can be differentiated from parasite membranes by their affinity for colloidal iron stains (*118*), or by their affinity for a variety of lectins (*121*). These newly developed staining procedures will make possible the resolution of the question of the origin of the membraneous profiles present in French pressure cell-prepared parasite preparations (*9, 36*). It will, of course, never be possible to eliminate host material completely from preparations of intraerythrocytic plasmodia because of the presence of food vacuoles in the parasites (*106*).

Soluble antigen in the plasma

Soluble plasmodial materials occur in the plasma of acutely infected monkeys (*47*), ducks (*143*), mice (*69*), chickens (*139*), and men (*90*). The material in the plasma of *P. knowlesi*-infected monkeys is active in CF tests, but does not stimulate protective immunity (*47*). Soluble parasite material in the plasma of mice infected with *P. berghei* (*69*) and chickens infected with *P. gallinaceum* (*139*) did stimulate protective immunity on injection into uninfected hosts. Cox et al. (*33*) considered the soluble parasite components in the serum to have a very broad antigenic specificity for intraerythrocytic parasites, but it is probable that autoantibodies to host lipids, which contaminated their preparations, were responsible for these results (*74, 120*). The soluble parasite components in chicken serum are proteinaceous. The soluble parasite components in the serum can be separated from the serum by precipitation with salts, by ultracentrifugation, and by a variety of column chromatography techniques (*140*). The parasite materials in the serum of chickens with *Plasmodium gallinaceum* have been used to sensitize latex for a tube latex agglutination test (*142*), and those in serum of rats will precipitate in gel with immune serum from rats (*163*). There are at least three soluble parasite components in the serum of infected chickens. These have molecular weights of 500 000–1 000 000, 150 000–250 000, and less than 70 000 (*130*). Soluble parasite products in the serum of humans with *Plasmodium-falciparum* infections have antigenic specificities similar to those of some of the components of the parasite body (*158, 159*). The soluble parasite components in the serum of *P. falciparum* infected humans are probably released at the time of rupture of schizonts and at the time of penetration of red cells by the merozoites (*157*).

Parasite-associated materials in the erythrocyte

Eaton (*46*) observed that immune serum from monkeys infected with *P. knowlesi* would agglutinate schizont-containing red cells. Uninfected erythrocytes from the blood were not agglutinated, nor were erythrocytes containing rings or more advanced trophozoites. Thus, these parasite materials must make their way into the red cell membrane from the inside, through the erythrocyte cytoplasm, as the parasites mature. The parasite materials in the membranes of erythrocytes containing *P. knowlesi* schizonts are variant antigens (*16*). Erythrocytes containing various other plasmodia have parasite associated materials in their cytoplasm (*3*), but as specific antiplasmodial antiserum will not usually agglutinate these erythrocytes (*14*), these materials probably do not extend through the membrane. Todorovic et al. (*141*) reported that fluorescein-labelled antibody specific for soluble antigens in the serum caused fluorescence and stimulated phagocytosis of erythrocytes of chickens with *P. gallinaceum* infection, and caused fluorescence of free merozoites. These results would suggest that the parasite-associated materials in or on the erythrocytes, and on the merozoites, and the soluble materials in the serum are antigenically at least similar. Unfortunately, this work was done with outbred chickens, and it is not possible to exclude the possibility that the results reported were caused by antibodies to blood group antigens rather than parasite antigens. Attempts to isolate the membrane-associated antigens for study do not appear to have been made.

ISOLATION OF STAGES FROM THE INVERTEBRATE

Gametocytes, oocysts, and other developmental stages

The recent report that immunization of chickens with formalin-treated or X-irradiated *P. gallinaceum*-infected red cells would inhibit oocyst formation in mosquitos that fed on the chickens, while barely affecting the asexual infections in the chickens (*56*), has suggested a possible new method of malaria control. Gwadz's (*56*) procedure of immunization used blood stage parasites, including gametocytes, to stimulate the production of antibodies, which when taken into the mosquito's gut with the blood meal presumably acted upon the gametes when they were released. Carter & Chen (*19*) obtained a higher degree of inhibition of oocyst formation by immunization with gametes. To obtain gametes, Carter & Chen (*19*) collected infected blood-containing gametocytes, washed the blood cells in a medium that inhibited gamete release, then suspended the gametocytes in a solution that stimulated gamete release. The gametes were collected by a series of centrifugations and the preparations were irradiated to suppress infectivity of contaminating asexual parasites. Gametocytes of *P. falciparum* can now be produced *in vitro* (*62, 128*), and this will possibly permit isolation of gametes of human plasmodia by techniques similar to those used by Carter & Chen (*19*).

Plasmodium berghei ookinetes may be isolated from mosquito midguts in large numbers (*154*). Midguts are dissected from mosquitos 9–12 or 18–24 hours after engorgement. A variety of antibiotics are added to the medium in which the midguts are suspended. The guts are ground by 5–10 strokes in a Ten Broeck tissue grinder and the homogenate digested with collagenase and hyaluronidase at 20–21°C for 45–60 min while being gently stirred. The digest is then centrifuged at 50 *g* for 5 min to remove gross debris, and the supernatant is saved. The pellet is resuspended and the process repeated a total of 5 times. The pooled supernatants are centrifuged at 500 *g* for 15 min to sediment the ookinetes. The ookinetes in the pellet are then further cleaned by gradient centrifugation. The ookinetes are morphologically intact and motile, but because of rather heavy contamination with microorganisms are not very suitable for culture. To obtain the mosquito stages of plasmodia uncontaminated with bacteria and yeasts, two courses of action may be followed; one course is to raise mosquitos free of living microorganisms (*144*), the other is to culture directly from the blood those stages that are normally in the mosquito (*20*). Mosquito cell cultures are commonly used as support for the cultured plasmodia. Sporozoites developed from oocysts in the presence of mosquito cells (*7*) and ookinetes developed from gametocytes in such systems (*4, 110*). Ookinetes will develop from gametocytes in cultures of fat head minnow epithelial cells also (*109*), but ookinete yields in cell culture systems are small, and further development does not occur (*122*).

Sporozoites

It is hoped some day, to obtain masses of sterile sporozoites from cultures inoculated with gametocyte-containing blood; this is not at present possible. The common sources of sporozoites for study are the salivary glands or whole bodies of infected mosquitos. A procedure for obtaining relatively small numbers of clean sporozoites involves dissecting the salivary glands from the mosquito, placing the freed glands under a coverslip in saline solution, and causing a current of fluid to pass under the coverslip by dropping saline on one side, and withdrawing it from the other with a capillary tube. The sporozoites leave the glands, enter the saline and are collected with it. Simple centrifugation permits concentration of the sporozoites in the saline (*31*). A procedure for obtaining relatively large numbers of sporozoites involves grinding whole mosquitos, or mosquito thoraxes or abdomens, suspended in tissue culture medium 199, with a loose fitting Teflon tissue grinder. After grinding, the heavier fragments are eliminated by centrifugation and then the sporozoites are separated from most of the remaining debris by centrifugation on a bovine serum albumin–Renografin gradient. The sporozoites are concentrated in the region of the gradient with a specific gravity of about 1.10 (*78*). Bosworth et al. (*10*) described a procedure for mass isolation of *Anopheles stephensi* salivary glands infected with sporozoites. Decapitated mosquitos are washed on a 22-gauge mesh plastic screen, then spread as a monolayer on a glass plate between tracks formed by feeler gauges of specific thicknesses which serve as spacers. A roller is passed over the tracks. The salivary glands are expelled from the bodies, but the bodies are not crushed because the spacers maintain an appropriate distance between the roller and the glass plate. The expelled glands may be washed from the bodies by screening, and collected for further processing to obtain sporozoites.

Inoculation with X-irradiated sporozoites induces immunity against sporozoite-induced infection, but attempts to isolate an immunogenic subfraction of the sporozoites have not succeeded (*98*).

CONCLUSIONS

Procedures are available for obtaining quantities of exoerythrocytic merozoites of the avian plasmodia, but we do not have satisfactory procedures for obtaining exoerythrocytic merozoites of mammalian plasmodia, nor do we have any procedures for obtaining the intercellular forms of exoerythrocytic stages of plasmodia.

A variety of procedures for obtaining intraerythrocytic plasmodia have been described. All have their uses and limitations. Continuous flow sonication will provide morphologically intact intraerythrocytic plasmodia free of host cell membranes, and spontaneous release of cultured schizonts provides erythrocytic merozoites. These two procedures together will provide free parasites of all the erythrocytic development stages. A variety of procedures are available for precipitating soluble products of the parasites from the plasma and from lysates of infected red cells, but little work has been done on the isolation of parasite-associated materials from the erythrocyte membrane. Recently developed techniques for culture of the erythrocytic stages of some species of plasmodia will make this stage easier to study. While procedures are available for obtaining preparations of many of the stages of plasmodia that occur in the mosquito, in particular gametes, ookinetes, and sporozoites, our inability to culture these stages for more than short periods remains a problem.

RÉSUMÉ

ISOLEMENT ET FRACTIONNEMENT DES CELLULES IMPALUDÉES

Ce document passe en revue les méthodes applicables à l'isolement des parasites du paludisme à partir des cellules hôtes, et à leur fractionnement. Les méthodes sont groupées en fonction du stade du parasite isolé; pour les cycles érythrocytaires, elles sont encore groupées par techniques. Certaines méthodes décrites concernent tous les stades parasitaires, tant chez les hôtes invertébrés que vertébrés. L'auteur indique les emplois et les limites des diverses méthodes et conclut que toutes ont leur utilité mais que, du point de vue morphologique, seules la production naturelle en culture et le traitement par ultrasons en continu fournissent de grandes quantités de parasites érythrocytaires intacts, exempts de membranes des cellules hôtes.

REFERENCES

1. AGENCY FOR INTERNATIONAL DEVELOPMENT. *Proposed strategy for expediting research and development for a malaria vaccine.* Washington, DC, United States Department of State, Office of Health, 1976.
2. AIKAWA, M. & COOK, R. T. *Experimental parasitology*, **31**: 67-74 (1972).
3. AIKAWA, M. ET AL. *American journal of pathology*, **79**: 285-294 (1975).
4. ALGER, N. E. *Nature*, **218**: 774 (1968).
5. ARNOLD, J. D. ET AL. *Journal of parasitology*, **55**: 597-608 (1969).
6. BAHR, G. F. Quantitative cytochemical study of erythrocytic stages of *Plasmodium lophurae* and *Plasmodium berghei*. In: Sadun, E. H. & Osborne, H. S., ed., *Research in malaria*. Special issue of *Military medicine*, vol. 131, pp. 1064-1070 (1966).
7. BALL, G. H. & CHAO, J. *Journal of parasitology*, **57**: 391-395 (1971).
8. BEAUDOIN, R. L. ET AL. *Experimental parasitology*, **36**: 355-359 (1974).
9. BECKWITH, R. ET AL. *Experimental parasitology*, **37**: 164-172 (1975).
10. BOSWORTH, A. B. ET AL. *Journal of parasitology*, **61**: 769-772 (1975).
11. BOWMAN, I. B. R. ET AL. *Experimental parasitology*, **9**: 131-136 (1960).
12. BRAY, R. S. & EL-NAHAL, H. M. S. *Nature (Lond.)*, **212**: 83 (1966).
13. BROWN, I. N. Immunological aspects of malaria infection. In: Dixon, E. J. & Kunkel, H. G. ed., *Advances in immunology*, vol. 11, New York, Academic Press, 1969, pp. 267-349.

14. BROWN, K. N. *Antigenic variation and immunity to malaria.* Amsterdam, ASP (Elsevier, Exerpta Medica, North Holland), 1974 (Ciba Foundation Symposium, No. 25, New Series).
15. BROWN, I. N. ET AL. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **60**: 3 (1966).
16. BROWN, I. N. ET AL. *Immunology*, **14**: 127-138 (1968).
17. BROWN, K. N. & HILLS, L. A. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **68**: 139-142 (1974).
18. BROWN, K. N. & TANAKA, A. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **69**: 350-353 (1975).
19. CARTER, R. & CHEN, D. H. *Nature (Lond.)*, **263**: 57-60 (1976).
20. CHAO, J. & BALL, G. H. *American journal of tropical medicine and hygiene*, **13** (Supplement): 181-192 (1964).
21. CHAVIN, S. I. Studies on the antigenic constituents of *Plasmodium berghei*. I. Immunologic analysis of the parasite constituents. II. Fractionation of the parasite constituents. In: Sadun, E. H. & Osborne, H. S., ed., *Research in malaria*. Special issue of *Military medicine*, vol. 131, pp. 1124-1136 (1966).
22. CHRISTOPHERS, S. R. & FULTON, J. D. *Annals of tropical medicine and parasitology*, **33**: 161-170 (1939).
23. CHOW, J. S. & KREIER, J. P. *Experimental parasitology*, **31**: 13-18 (1972).
24. COATNEY, G. R. ET AL. *The primate malarias.* Bethesda, United States Department of Health, Education, and Welfare, 1971.
25. COGGESHALL, L. T. & EATON, M. D. *Journal of experimental medicine*, **67**: 871-882 (1938).
26. COHEN, S. ET AL. *In vitro* studies of malaria antibody. In: Sadun, E. H. & Moon, A. P., ed., *Basic research in malaria*, Special issue of *Proceedings of the Helminthological Society of Washington*, vol. 39, pp. 231-237 (1972).
27. COOK, R. T. & AIKAWA, M. *Science*, **171**: 1175-1176 (1971).
28. COOK, R. T. ET AL. The isolation and fractionation of *Plasmodium knowlesi*. In: Sadun, E. H. & Moon, A. P., ed., *Experimental malaria*. Special issue of *Military medicine*, vol. 134, pp. 866-884 (1969).
29. COOK, R. T. ET AL. *Comparative biochemistry and physiology*. **39B**: 897-911 (1971).
30. CORRADETTI, A. ET AL. *Bulletin of the World Health Organization*, **35**: 802-805 (1966).
31. CORRADETTI, A. ET AL. *Bulletin of the World Health Organization*, **30**: 747-750 (1964).
32. COX, F. E. G. *Nature (Lond.)*, **252**: 268 (1974).
33. COX, H. W. ET AL. *American journal of tropical medicine and hygiene*, **17**: 13-18 (1968).
34. D'ANTONIO, L. E. *Experimental parasitology*, **31**: 75-81 (1972).
35. D'ANTONIO, L. E. *Journal of the American Osteopathic Association*, **74**: 649-652 (1974).
36. D'ANTONIO, L. E. & SILVERMAN, P. H. *Science*, **171**: 1176 (1971).
37. D'ANTONIO, L. E. ET AL. *Proceedings of the Society for Experimental Biology and Medicine*, **123**: 30-34 (1966).
38. D'ANTONIO, L. E. ET AL. Serological evaluation of the specificity and sensitivity of purified malaria antigens prepared by a new method. In: Sadun, E. H. & Osborn, H. S., ed., *Research in malaria*. Special issue of *Military medicine*, vol. 131, pp. 1152-1156 (1966).
39. DAVIS, B. D. *Journal of immunology*, **58**: 269-281 (1948).
40. DAVIS, A. G. ET AL. *Experimental parasitology*, **19**: 1-8 (1966).
41. DENNIS, E. D. ET AL. *Parasitology*, **71**: 475-481 (1975).
42. DESOWITZ, R. S. *Experimental parasitology*, **38**: 6-13 (1975).
43. DIGGS, C. L. *Experimental parasitology*, **19**: 237-248 (1966).
44. DOBY, J. M. & BARKER, R. *Comptes rendus des séances de la Société de Biologie*, **170**: 661-665 (1976).
45. DULANY, A. D. & STRATMAN-THOMAS, W. K. *Journal of immunology*, **39**: 247-255 (1940).
46. EATON, M. D. *Journal of experimental medicine*, **67**: 857-870 (1938).
47. EATON, M. D. *Journal of experimental medicine*, **69**: 517-553 (1939).
48. EL-NAHAL, H. M. S. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **61**: 7-9 (1967).
49. FIFE, E. H., JR *Experimental parasitology*, **30**:132-163 (1971).
50. FIFE, E. H., JR *Experimental parasitology*, **31**: 136-152 (1972).
51. FIFE, E. H., JR ET AL. *In vitro* and *in vivo* studies on a lytic factor isolated from *Plasmodium knowlesi*. In: Sadun, E. H. & Moon, A. P., ed., *Basic research in malaria*. Special issue of *Proceedings of the Helminthological Society of Washington*, vol. 39, pp. 373-382 (1972).
52. FERREHEE, J. W. & GEIMAN, Q. M. *Journal of infectious diseases*, **78**: 173-179 (1946).
53. FULTON, J. D. & GRANT, P. T. *Biochemical journal*, **63**: 274-282 (1956).
54. GEIMAN, Q. M. & MEAGHER, M. *Nature (Lond.)*, **215**: 437-439 (1967).
55. GRAVELY, S. M. & KREIER, J. P. *Tropenmedizin und Parasitologie*, **25**: 198-206 (1974).
56. GWADZ, R. W. *Science*, **193**: 1150-1151 (1976).
57. HAMBURGER, J. & KREIER, J. P. *Infection and immunity*, **12**: 339-345 (1975).
58. HAMBURGER, J. & KREIER, J. P. *Experimental parasitology*, **40**: 158-169 (1976).

59. HAMBURGER, J. & KREIER, J. P. *Tropenmedizin und Parasitologie*, **27**: 385-390 (1976).
60. HAMBURGER, J. & ZUCKERMAN, A. *Experimental parasitology*, **39**: 460-478 (1976).
61. HAMBURGER, J. & ZUCKERMAN, A. *Experimental parasitology*, **39**: 479-495 (1976).
62. HAYNES, J. D. ET AL. *Nature (Lond.)*, **263**: 767-769 (1976).
63. HEIDELBERGER, M. & MAYER, M. M. *Science*, **100**: 359-360 (1944).
64. HOLBROOK, T. W. ET AL. *Journal of parasitology*, **60**: 348-354 (1974).
65. HOLBROOK, T. W. ET AL. *Journal of parasitology*, **62**: 670-675 (1976).
66. HOMEWOOD, C. A. & NEANE, K. D. *Annals of tropical medicine and parasitology*, **70**: 249-251 (1976).
67. HUFF, C. G. *American journal of tropical medicine and hygiene*, **13**: 171-177 (1964).
68. HUFF, C. G. *Experimental parasitology*, **24**: 383-421 (1969).
69. JERUSALEM, C. *Zeitschrift für Tropenmedizin und Parasitologie*, **19**: 171-180 (1968).
70. JERUSALEM, C. *Zeitschrift für Tropenmedizin und Parasitologie*, **20**: 430-439 (1969).
71. JERUSALEM, C. & ELING, W. *Bulletin of the World Health Organization*, **40**: 807-818 (1969).
72. JERUSALEM, C. ET AL. *Journal of immunology*, **107**: 260-268 (1971).
73. KILBY, V. A. A. & SILVERMAN, P. H. *American journal of tropical medicine and hygiene*, **18**: 836-859 (1969).
74. KREIER, J. P. ET AL. *Experimental parasitology*, **19**: 155-162 (1966).
75. KREIER, J. P. ET AL. *Tropenmedizin und Parasitologie*, **26**: 9-18 (1975).
76. KREIER, J. P. ET AL. *Tropenmedizin und Parasitologie*, **27**: 82-88 (1976).
77. KREIER, J. P. ET AL. *American journal of tropical medicine and hygiene*, **14**: 529-532 (1965).
78. KRETTLI, A. ET AL. *Journal of protozoology*, **20**: 662-665 (1973).
79. KROTOSKI, W. A. ET AL. *Journal of parasitology*, **60**: 334-347 (1974).
80. LANGER, B. W. ET AL. *Experimental parasitology*, **20**: 68-76 (1967).
81. LANGRETH, S. G. & TRAGER, W. *Journal of protozoology*, **20**: 606-613 (1973).
82. LUND, M. N. & POWERS, K. G. *Annals of tropical medicine and parasitology*, **70**: 284-291 (1976).
83. MANNWEILER, E. & OELERICH, S. *Zeitschrift für Tropenmedizin und Parasitologie*, **20**: 265-279 (1969).
84. MARTIN, W. J. ET AL. *Nature, New Biology*, **233**: 260-261 (1971).
85. MATHEWS, H. M. ET AL. *American journal of tropical medicine and hygiene*, **24**: 417-422 (1975).
86. MAYER, M. M. & HEIDELBERGER, M. *Journal of immunology*, **54**: 89-102 (1946).
87. MCALISTER, R. O. Fractionation of the serologically reactive antigens of *Plasmodium falciparum*. In: Sadun, E. H. & Moon, A. P., ed., *Basic research in malaria*. Special issue of *Proceedings of the Helminthological Society of Washington*, vol. 39, pp. 554-562 (1972).
88. MCALISTER, R. O. & GORDON, D. M. *Journal of parasitology*, **62**: 664-669 (1976).
89. MCGREGOR, I. A. *International review of tropical medicine*, **4**: 1-52 (1971).
90. MCGREGOR, I. A. ET AL. *Lancet*, **1**: 881-884 (1968).
91. MEUWISSEN, J. H. E. T. ET AL. *Bulletin of the World Health Organization*, **46**: 771-782 (1972).
92. MILLER, L. H. ET AL. *Journal of immunology*, **114**: 1237-1242 (1975).
93. MILLER, L. H. & CHIEN, S. *Experimental parasitology*, **29**: 451-456 (1971).
94. MITCHELL, G. H. ET AL. *International journal for parasitology*, **3**: 443-445 (1973).
95. MITCHELL, G. H. ET AL. *Nature (Lond.)*, **252**: 311-313 (1974).
96. MITCHELL, G. H. ET AL. *Immunology*, **29**: 397-407 (1975).
98. NUSSENZWEIG, R. S. ET AL. *American journal of tropical medicine and hygiene*, **21**: 722-728 (1972).
99. PORTER, J. A., JR & YOUNG, M. D. Susceptibility of Panamanian primates to *Plasmodium vivax*. In: Sadun, E. H. & Osborne, H. S., ed., *Research in malaria*. Special issue of *Military medicine*, vol. 131, pp. 952-958 (1966).
100. PRIOR, R. B. & KREIER, J. P. *Experimental parasitology*, **32**: 239-243 (1972).
101. PRIOR, R. B. & KREIER, J. P. Isolation of *Plasmodium berghei* by use of a continuous-flow ultrasonic system: A morphological and immunological evaluation. In: Sadun E. H. & Moon A. P., ed., *Basic research in malaria*. Special issue of *Proceedings of the Helminthological Society of Washington*, vol. 39, pp. 563-574 (1972).
102. PRIOR, R. B. & KREIER, J. P. *Ohio journal of science* (in press).
103. PRIOR, R. B. ET AL. *Journal of parasitology*, **59**: 200-201 (1973).
104. RICHARDS, W. H. G. & WILLIAMS, S. G. *Annals of tropical medicine and parasitology*, **67**: 249-250 (1973).
105. ROCK, R. C. ET AL. *Comparative biochemistry and physiology*, **33 B**: 425-437 (1971).
106. RODZINSKA, M. A. & TRAGER, W. *Journal of protozoology*, **4**: 190-199 (1957).
107. ROGERS, T. E. Analytical serology of protozoa. In: Kwapinski, J. B. C. ed., *Analytical serology of microorganisms*. New York, Interscience Publishers, John Wiley and Sons, 1969, pp. 549-637.
108. RUTLEDGE, L. C. & WARD, R. A. *Experimental parasitology*, **20**: 167-176 (1967).

109. ROSALES-RONQUILLO, M. C. ET AL. *Journal of parasitology*, **60**: 1039-1040 (1974).
110. ROSALES-RONQUILLO, M. C. & SILVERMAN, P. H. *Journal of parasitology*, **60**: 819-824 (1974).
111. ROWLEY, P. T. ET AL. *Journal of laboratory and clinical medicine*, **70**: 933-938 (1967).
112. SADUN, E. H. & GORE, R. W. *Experimental parasitology*, **23**: 277-285 (1968).
113. SAUL, K. & KREIER, J. P. *Tropenmedizin und Parasitologie* (in press).
114. SCHEIBEL, L. W. & MILLER, J. *Journal of parasitology*, **55**: 825-829 (1969).
115. SCHENKEL, R. H. ET AL. *Journal of parasitology*, **61**: 549-550 (1975).
116. SCHENKEL, R. H. ET AL. *Bulletin of the World Health Organization*, **48**: 597-604 (1973).
117. SEED, T. M. AL. ET *Zeitschrift für Tropenmedizin und Parasitologie*, **24**: 525-535 (1973).
118. SEED, T. M. ET AL. *Journal of protozoology*, **20**: 603-605 (1973).
119. SEED, T. M. ET AL. *Infection and immunity*, **9**: 750-761 (1974).
120. SEED, T. M. & KREIER, J. P. Autoimmune reactions in chickens with *Plasmodium gallinaceum* infection: The isolation and characterization of a lipid from trypsonized erythrocytes which reacts with serum from acutely infected chickens. In: Sadun, E. H. & Moon, A. P., ed., *Experimental malaria*. Special issue of *Military medicine*, vol. 134, pp. 1220-1227 (1969).
121. SEED, T. M. & KREIER, J. P. *Infection and immunity*, **14**: 1139-1347 (1976).
122. SHAPIRO, M. ET AL. *Journal of parasitology*, **61**: 1105-1106 (1975).
123. SHERMAN, I. W. *Journal of protozoology*, **11**: 409-417 (1964).
124. SHERMAN, I. W. & HULL, R. W. *Journal of protozoology*, **7**: 409-416 (1960).
125. SHUNGU, D. M. & ARNOLD, J. D. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **65**: 684-685 (1971).
126. SIDDIQUE, W. A. ET AL. *American journal of tropical medicine and hygiene*, **23**: 1015-1018 (1974).
127. SIMPSON, G. L. ET AL. *Nature (Lond.)*, **247**: 304-306 (1974).
128. SMALLEY, M. E. *Nature (Lond.)*, **264**: 271-272 (1976).
129. SMALLEY, M. E. & BUTCHER, G. A. *International journal for parasitology*, **5**: 131-132 (1975).
130. SMITH, A. R. ET AL. *Experimental parasitology* **31**: 120-125 (1972).
131. SODEMAN, W. A. JR & MEUWISSEN, J. H. E. T. *Journal of parasitology*, **52**: 23-25 (1966).
132. SPEER, C. A. ET AL. *Journal of protozoology*, **23**: 437-442 (1976).
133. SPEER, C. A. ET AL. *Journal of parasitology* **62**: 657-663 (1976).
134. SPIRA, D. & ZUCKERMAN, A. *Science*, **137**: 536-537 (1962).
135. SPIRA, D. & ZUCKERMAN, A. Recent Advances in the antigenic analysis of plasmodia. In: Sadun, E. H. & Osborn, H. S., ed., *Research in malaria*. Special issue of *Military medicine*, vol. 131, pp. 1117-1123 (1966).
136. STAUBER, L. A. & WALKER, H. A. *Proceedings of the Society for Experimental Biology and Medicine*, **63**: 223-227 (1946).
137. STAUBER, L. A. ET AL. *Journal of infectious diseases*, **89**: 31-34 (1951).
138. STEIN, B. & DESOWITZ, R. S. *Bulletin of the World Health Organization*, **30**: 45-49 (1964).
139. TODOROVIC, R. ET AL. *Annals of tropical medicine and parasitology*, **61**: 117-124 (1967).
140. TODOROVIC, R. ET AL. *American journal of tropical medicine and hygiene*, **17**: 685-694 (1968).
141. TODOROVIC, R. ET AL. *American journal of tropical medicine and hygiene*, **17**: 695-701 (1968).
142. TODOROVIC, R. ET AL. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **62**: 58-68 (1968).
143. TORREY, J. C. & KAHN, M. C. *Journal of immunology*, **62**: 41-48 (1949).
144. TRAGER, W. *American journal of hygiene*, **22**: 18-25 (1935).
145. TRAGER, W. *Journal of experimental medicine*, **92**: 349-366 (1950).
146. TRAGER, W. *Journal of protozoology*, **1**: 231-237 (1954).
147. TRAGER, W. & JENSEN, J. B. *Science*, **193**: 673-675 (1976).
148. TRAGER, W. ET AL. Viability and fine structure of extracellular *Plasmodium lophurae* prepared by different methods. In: Sadun, E. H. & Moon, A. P., ed., *Basic research in malaria*. Special issue of *Proceedings of the Helminthological Society of Washington*, vol. 39, pp. 220-230 (1972).
149. TRAGER, W. ET AL. *Proceedings of the Society for Experimental Biology and Medicine*, **75**: 766-771 (1950).
150. TRIGG, P. I. ET AL. *Parasitology*, **71**: 199-209 (1975).
151. VERAIN, A. & VERAIN, A. *Compte rendus des séances de la Société de Biologie et de ses filiales*, **150**: 1189-1190 (1956).
152. WALTER, R. D. *Zeitschrift für Tropenmedizin und Parasitologie* **19**: 415-426 (1968).
153. WARD, P. A. & CONRAN, P. B. Immunopathologic studies of simian malaria. In: Sadun, E. H. & Osborn, H. S., ed., *Research in malaria*. Special issue of *Military medicine*, vol. 131, pp. 1225-1232 (1966).
154. WEISS, M. M. & VANDERBERG, J. P. *Journal of protozoology*, **23**: 547-551 (1976).
155. WHO Technical Report Series, No. 579, 1975 (*Developments in malaria immunology*: report of a WHO Scientific Group).

156. WILLIAMSON, J. & COVER, B. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **60**: 425-427 (1966).
157. WILSON, R. J. M. & BARTHOLOMEW, R. K. *Parasitology*, **71**: 183-192 (1975).
158. WILSON, R. J. M. ET AL. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **69**: 453-459 (1975).
159. WILSON, R. J. M. ET AL. *International journal for parasitology*, **3**: 511-520 (1973).
160. YU, D. T. Y. ET AL. *Journal of immunology*, **110**: 1615-1622 (1973).
161. ZUCKERMAN, A. *Bulletin of the World Health Organization*, **40**: 55-66 (1969).
162. ZUCKERMAN, A. Malaria of Lower Mammals. In: Jackson, G. J. et al., ed., *Immunity to parasitic animals II*. New York, Appleton-Century-Crofts, 1970.
163. ZUCKERMAN, A. ET AL. *Experimental parasitology*, **24**: 299-312 (1969).
164. ZUCKERMAN, A. & RISTIC, M. Blood parasite antigens and antibodies. In: Weinman, D. & Ristic, M., ed., *Infectious blood diseases of man and animals*. New York, Academic Press, 1968, pp. 79-122.
165. ZUCKERMAN, A. ET AL. *Bulletin of the World Health Organization*, **37**: 431-436 (1967).