INFORMATION TO USERS

This dissertation was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

University Microfilms

300 North Zeeb Road
Ann Arbor, Michigan 48106

A Xerox Education Company

PLASMODIUM BERGHEI FREED FROM HOST ERYTHROCYTES BY A CONTINUOUS-FLOW ULTRASONIC SYSTEM: A MORPHOLOGICAL AND IMMUNOLOGICAL STUDY

DISSERTATION

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

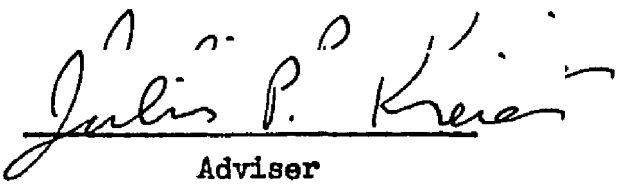
By

Richard Bruce Prior, B.A., M.Sc.

* * * * *

The Ohio State University
1972

Approved by



Adviser

ACKNOWLEDGMENTS

I would sincerely like to thank my adviser, Dr. Julius P. Kreier, for his support, guidance, and advice during the course of this investigation. Also, I would like to thank Dr. Robert C. McMaster for acting as my co-adviser and for making many helpful suggestions in the area of ultrasonics.

I am deeply grateful to Dr. Robert Pfister and Mr. Owen Kindig for their assistance and advice in the electron microscopy. In addition, I would like to acknowledge my friends and associates for their interest and cooperation in this study. In particular, I would like to thank Cecil L. Rhodes, Thomas Seed, and John Mansfield for their helpful suggestions.

Finally, I would like to thank my lovely wife, Marthenia, for her interest and patience in this study and gratefully acknowledge her effort in typing this manuscript.

VITA

May 28, 1941	Born - Salem, Ohio
1966	B.A., The Ohio State University, Columbus, Ohio
1966-1971	Research Specialist, Department of Welding Engineering, The Ohio State University, Columbus, Ohio
1968	M.Sc., The Ohio State University, Columbus, Ohio
1971	Teaching Associate, Department of Microbiology, The Ohio State University, Columbus, Ohio
1972	Research Associate, Department of Microbiology, The Ohio State University, Columbus, Ohio

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Malaria is a protozoan disease which is transmitted to man by bites of mosquitos. In man, four species of the protozoan Plasmodium cause malaria. They are Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, and Plasmodium ovale. Although the incidence of malaria has been greatly reduced in the United States by control of the mosquito vector, malaria is still a very serious disease in other parts of the world. It is estimated that approximately 3 million deaths each year are caused by malaria, thus making it the greatest killer of human beings of any infectious disease. Brown (1969) points out that, in addition to causing high mortality, malaria reduces the vigor of communities and, by its continued presence, causes retardation of economic and social growth. Ironically, man to some extent has helped propagate malaria in certain geographical areas. For example, recent governmental bans on the use of DDT have caused an increase in malaria. Also Oricens, et al. (1970) point out that the recent bombings of Viet Nam have left countless bomb craters which serve as ideal breeding places for the mosquitos during the rainy season. So it is that malaria is still a very serious problem, and much research is needed if malaria is to be controlled.

The exact routes along which research should be directed to conquer malaria are somewhat controversial. However, it is generally agreed among malariologists that a thorough analysis of the parasites' antigenic structure could possibly lead to the development of effective

vaccines and could possibly lead to the development of a sero-diagnostic tool. The latter would be most helpful especially in these days of rapid travel when one may contract the disease in another country and return to the United States before the disease becomes evident. In addition, knowledge of the parasites' metabolism could lead to the cultivation of the parasites in artificial media as well as to the development of effective anti-malarials. However, before many of these studies can be carried out, relatively clean preparations of plasmodia, free of host erythrocytes, are needed. For this reason, many attempts have been made to separate plasmodia from the erythrocytes.

Investigators have tried various physical, chemical, and immunolytic means to effect separation; but they reported loss or change of the antigenic structure, low reproducibility of test results, damage to the parasites, or incomplete separation. Therefore, in an effort to overcome the problems encountered with other means of separation, some investigators have tried to use high-frequency sonic waves to effect complete separation of plasmodia from host erythrocytes (Verain and Verain, 1956; Kreier et al., 1965; Rutledge et al., 1967; Weiss, 1967; and Prior, 1968). Since ultrasonic energy is mechanical in nature, disruption of malaria-infected erythrocytes by ultrasound should not adversely affect the chemical composition of the parasites. Although each investigator reported release of malaria parasites from erythrocytes by ultrasound, the batch treatment used by most investigators degraded many free parasites, thereby precluding the use of such systems to obtain large quantities of free parasites.

In the study to be reported, ultrasonic energy was used to free malaria parasites from their host erythrocytes, but a continuous-flow system of sonication was designed and built to replace the batch system of sonication used in previous studies. In the continuous-flow system of sonication, each infected erythrocyte receives approximately equal exposure to ultrasonic energy, and the parasites freed from their host erythrocytes are then rapidly removed from the sonic field and its destructive forces.

This research was divided into four basic phases. These four phases were as follows:

Phase I: The development of a suitable continuous-flow ultrasonic system which would yield large quantities of free malaria parasites with a minimum amount of debris. The system should be easy to construct, inexpensive, and simple to operate to encourage its use by other laboratories.

Phase II: The development of a suitable parasite separation technique. Such a procedure should separate those parasites freed by ultrasound from undesirable components such as ghost cells and membrane fractions and recover a large percentage of the free parasites. The addition of foreign chemicals should be avoided if possible.

Phase III: The morphological examination of the free parasite pellet obtained in Phase II. Such an examination should include the various light microscopic techniques

but must include low-power thin-section electron microscopy. The criterion of success of Phases I and II is the absence of host erythrocyte membranes in the parasite preparation.

Phase IV: A general antigenic analysis of the free-parasite preparations. Such analysis should determine the specificity of the antigen for antisera as well as provide a critical method for appraising the purity of the antigen. The complement-fixation test is the test of choice as it is a highly standardized, very sensitive, and widely used test in malaria research. The antigenic analysis should also include a study on the stability of the new antigen to determine optimum conditions of storage.

The successful completion of these four phases of research provides a basic model system for further research. Hopefully, the system can be adapted to utilize other parasites such as Plasmodium knowlesi and Plasmodium falciparum, as these parasites provide important antigens for use in human malaria research.

ORGANIZATION OF DISSERTATION

This dissertation is basically organized in the same way as the research plan described in the previous section. Emphasis is placed on ultrasound, and its controlled use for the freeing of Plasmodium berghei from the host erythrocyte. A brief literature review (Chapter I) is included to acquaint the reader with ultrasound, its various modes of actions in liquids, and its present use in malaria research. Essentially, this review is an edited version of the one presented previously (Prior, 1968) with pertinent current literature added.

In Chapter II, the materials and methods used in this research are presented. The continuous-flow sonication system is described, and special attention is given to the continuous-flow sonication chamber which was developed specifically for this study. A typical run is discussed thoroughly, including the preparation of the specimen and the mechanics of establishing testing parameters for the system. This is followed by a detailed description of the actual sonication of the specimen. The technique used to harvest the free parasites is then presented. The latter part of Chapter II deals with the various procedures used to prepare the free parasites for microscopic and serologic examination. The method used to estimate the infectivity of the free parasites completes this chapter.

The results and discussion section (Chapter III) is divided into two major parts. The first part is an analysis of the light and

electron micrographs of the free parasite preparation. The second part presents the results obtained by the complement-fixation tests performed. The discussion section is incorporated in the results to eliminate referring back to the various photographs and tables.

Chapter IV is a brief summary of the results obtained, and emphasis is placed on tying together the results so the system developed and used in this study may be adequately evaluated. Chapter V lists the conclusions of this study and is followed by a list of recommendations for further research.

Preceding the Bibliography is the Appendix which lists alphabetically the various chemicals and reagents used in this study. Brief instructions follow some of the compounds listed to explain how these compounds were made.

CHAPTER I

L I T E R A T U R E R E V I E W

CHARACTERISTICS OF ULTRASOUND AND PHENOMENA ASSOCIATED WITH ULTRASOUND

Ultrasonic Energy

Ultrasound is defined as vibration of the same physical nature as sound but with frequencies above the range of human hearing. Accordingly, the frequency range of ultrasound is quite wide, beginning at about 20,000 Hz (cycles per second) with 1,000,000,000 Hz the highest known frequency attained (Carlin, 1964).

The ultrasonic waves or vibrations are mechanical in nature. The sound waves are longitudinal as they are propagating through a liquid medium, and the velocity of the sound waves in the liquid depends on the compressibility and density of the medium through which the waves travel (El'piner, 1964). The wavelength of the sound wave is inversely proportional to the frequency and the relationship between frequency, velocity, and wavelength is expressed as:

$$\lambda = \frac{c}{f}$$

where:

λ = wavelength
c = propagation velocity of sound wave
f = frequency of sound wave

From this expression, it is evident that wavelength is dependent on both the frequency and the velocity of the sound wave.

Ultrasonic energy has been used in both industrial and biological applications. Depending upon the amplitude of the ultrasonic waves, destructive or nondestructive phenomena occur. Low-amplitude pulses are used in both industrial and medical applications to detect flaws or abnormalities within a specimen without damaging it. High-amplitude waves are used in industry for cleaning, soldering and brazing, welding, drilling, emulsification, etc. (Carlin, 1964). High-amplitude waves are also used to effect cellular disruption and such waves will be the primary concern in this review.

ULTRASONIC APPLICATIONS

Frequency (kHz)	Process
3-20	Gas processing, defoaming, precipitation, drilling, soldering, cleaning, etc., degassing
20-100	Depolymerization (reported at various frequencies)
40	Cleaning and industrial applications
150	Mixing oil and water
300	Resonance testing (lowest frequency)
300	Physical and biological effects
330	Virus treatment
450	Dispersion of mercury
200-2,000	Halogen-silver-gelatin emulsions
500-15,000	Pulsed testing-effects on zinc while solidifying, luminescence
1,000,000	Highest known frequency attained

Frequency also plays an important role in ultrasonic applications. As mentioned previously, wavelength varies inversely with frequency. The preceding table modified from Carlin (1964) briefly summarizes some ultrasound studies and shows frequencies commonly used and their associated applications. However, Carlin (1964) states that it has not been proved that most of these applications are frequency-sensitive, except so far as frequency and cavitation are related. The frequency used in this study to produce acoustic cavitation and concomitant red cell lysis was 20,000 Hz.

Acoustic Cavitation

Sound waves of moderate intensity propagating through a liquid produce a physical phenomenon called acoustic cavitation. Cavitation is the generation within the liquid of small bubbles whose motions bring about drastic effects such as chemical reactions, erosion, emission of light, and radiation of sound (Flynn, 1964). The intensity of ultrasonically-produced cavitation is affected by various factors such as static pressure externally applied to the liquid, temperature of the liquid, size of the bubble, frequency of the applied sound field, intensity of the sound field, vapor pressure of the liquid, viscosity of the liquid, and density of the liquid to mention just a few (Frederick, 1965). In fact, most of these factors are interrelated. For example, maximum bubble size increases if the applied sound intensity is increased, and the bubble size decreases if the frequency of the applied sound field is increased. The primary reason for considering these

factors is that cavitation seems to be the single greatest cause of cellular disruption (Frederick, 1965; Ackerman, 1953; and Weiss, 1967).

The phenomenon of cavitation in a liquid exposed to acoustic waves of high amplitude has been the concern of investigators for many years. Lord Rayleigh (1917) was the first to propose theoretically a model which would explain how cavitation brings about its drastic effects. Lord Rayleigh (1917) suggested that the pressure inside the bubble was less than the ambient pressure in the liquid. This pressure remains constant as the cavity contracts from its original size, and the inward speed of the cavity wall increases without limit as the radius of the cavity approaches zero. Therefore, the cavity cannot be in equilibrium with the liquid, and collapse must start immediately. Rayleigh (1917) also pointed out that before complete collapse of the cavity, the pressure near the boundary of the cavity becomes very great. But the pressure at the boundary itself is zero as long as the motion of the cavity is free. This type of cavity is commonly referred to as the Rayleigh Cavity.

Other models of cavitation bubbles and their motions have been proposed. Flynn (1964) describes two additional theoretical models to represent better the remaining motions, not described by the Rayleigh cavity, of a gas-vapor bubble which was initially at rest in a liquid. Flynn (1964) suggests such a bubble may be represented by:

1. A transient cavity if, on contraction from some maximum size, its initial motion approximates that of a Rayleigh cavity and its inward speed increases until a rapid rise of pressure within the cavity arrests its inward motion.

2. A stable cavity if it oscillates nonlinearly about its equilibrium radius.

Flynn (1964) also describes two different types of transient cavities. The first type is a gaseous transient cavity in which the relative amounts of gas and vapor remain fixed during a pulsation, and the second type is a vaporous transient cavity in which the amounts of vapor and gas may change during a pulsation.

Flynn (1964) suggests that the terms "cavitation event," "cavitation field," and "cavitation activity measure" be restricted to bubble fields in which transient cavities occur; and this author defines cavitation activity measure as any activity measure based on a physical effect brought about by transient cavities only. But Flynn (1964) points out that bubbles resembling stable cavities can rupture the cell walls of E. coli as reported by Hughes and Nyborg. Since cell rupture is a physical effect, this effect thus qualifies as an activity measure associated with stable bubble fields. With this exception, Flynn (1964) still restricts the terms mentioned above to those situations where transient cavities produce such physical effects as erosion, emission of light, and chemical reactions.

Since it is the motions (collapses of small bubbles generated in a acoustically-irradiated liquid) which bring about many physical effects such as cell rupture, many investigators have tried to measure the amount of acoustic pressure necessary to induce these bubbles. While a discussion of this subject is beyond the scope of this review, Prior (1968) includes a thorough discussion of the measurement of the thresholds of cavitation as well as the measurement of cavitation. Prior (1968) also includes a discussion of the other effects observed in a

cavitating ultrasonic field. Such effects include thermal and chemical phenomena.

Figure 1 is a brief outline showing some effects produced by acoustic cavitation which itself is produced by high-amplitude ultrasonic waves. Suppression of acoustic cavitation results in the concomitant reduction or loss of these effects. Measurement of any one of these effects usually indicates the amount of acoustic cavitation present. Cavitation must be controlled if reproducible results are to be obtained. Many factors influence cavitation, thus making it extremely difficult to control. For example, gas content of the liquid will greatly affect the amount of cavitation present. In this study, acoustic cavitation was indirectly determined by using the very effect the system was designed to produce, i.e. red cell breakage. Power levels were adjusted to achieve approximately 80 percent red cell breakage. Accordingly, adjustment of the power levels of any other system to achieve 80 percent red cell breakage should recreate a sound field nearly identical to the one used in this study.

ULTRASOUND AND ITS USE IN MALARIA RESEARCH

Erythrocyte Destruction by Ultrasonic Energy

Early investigators found that erythrocytes were rapidly destroyed when exposed to ultrasonic energy of moderate intensity (Wood and Loomis, 1927; Chambers and Gaines, 1932). Johnson (1929) found that when external pressures of about 65 pounds per square inch were placed on a blood suspension, no destructive effects occurred; but when the

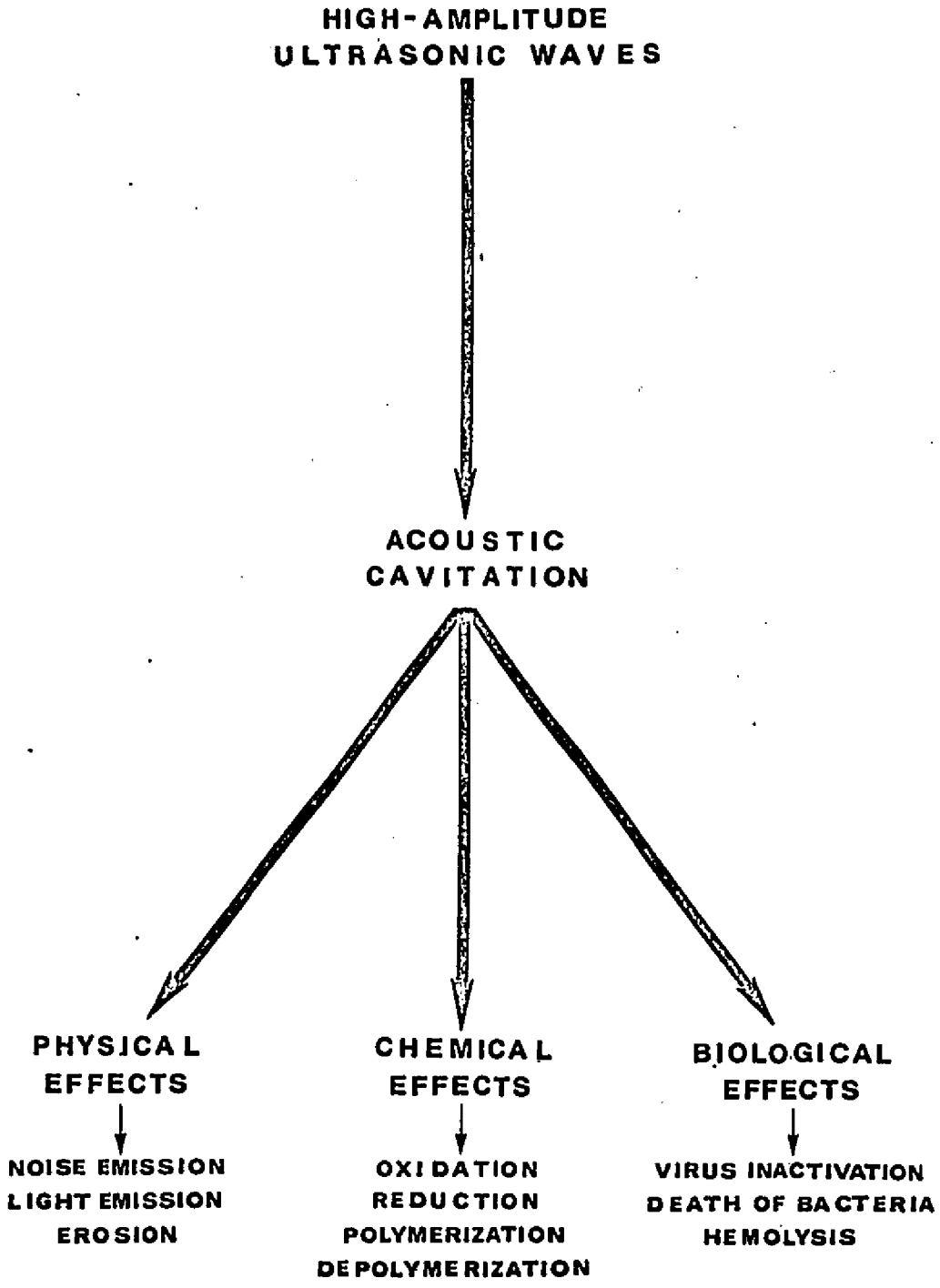


Fig. 1. Outline showing some effects produced by acoustic cavitation.

pressure was removed, hemolysis followed. Johnson (1929) attributed the destructive effects of sound on red blood cells to the formation of minute bubbles of gas within the liquid. The external pressure suppressed the formation of the bubbles; thus no hemolysis occurred. Weiss (1967) also found that increasing the static pressure on an erythrocyte suspension exposed to acoustic energy greatly reduced the rate of erythrocyte destruction. As pointed out previously, static pressure on a liquid affects the threshold level for the occurrence of cavitation. The result of increasing the static pressure is a decrease in bubble formation resulting in a reduction of hemolysis. Therefore, it is apparent cavitation plays a very important role in red cell destruction.

The exact role cavitation plays in effecting cell destruction has not been thoroughly worked out. Chambers and Gaines (1932), Ackerman (1953), and Hughes and Nyborg (1962) have proposed that large local shearing stresses due to shock fronts found in the vicinity of cavitating bubbles mechanically rupture cell membranes. Grabar (1953) suggested that violent motions occur close to the cavitation bubble and rather weak motions occur several bubble diameters away. Therefore, the part of the cell membrane adjacent to the bubble will incur large motions relative to the remainder of the cell membrane. Thus, the membranes become distorted resulting in a mechanical tearing of the cells exposed to a cavitating ultrasonic field (Grabar, 1953). Smith (1935) suggested the strong local stresses caused by the pulsating bubbles strain cells beyond their elastic limit in the vicinity of cavitation bubbles. Ackerman (1953) postulated that cellular breakdown due to cavitation was

due to extremely small bubbles which oscillate with the pressure field producing large displacement gradients in the cell walls.

Aside from the direct effects cavitation causes in erythrocyte destruction, studies have been made to seek a frequency effect (Angerer et al., 1951; Ackerman, 1953; Lehmann, 1953; Lombard, 1955; Ackerman and Lombard, 1955; Ackerman and Proctor, 1956; Binstock and Ackerman, 1959; Binstock, 1960; and Weiss, 1967). These investigators reported certain frequencies at which erythrocytes appeared to be more sensitive to ultrasound; but, as pointed out, one should be cautious since these results may be more a reflection of indirect frequency effects on cavitation than direct frequency-dependent breakdown of cells (Carlin, 1964). Ackerman and Proctor (1956) demonstrated surface resonant modes for air bubbles in a water column subjected to cavitating ultrasonic fields. These investigators point out that the primary action of cavitation bubbles probably was to generate a sound field which was capable of exerting cellular surface vibration modes. Furthermore, Ackerman (1952a) observed certain optimum frequencies between 300 kHz and 600 kHz for breaking bovine, rabbit, sheep, and human erythrocytes exposed to ultrasonic energy. Ackerman (1952a) attributed these results to certain mechanical resonances involving the cell surfaces.

Besides the direct action of cavitation and cellular resonances per se, Ackerman (1952b) studied the mechanical fragilities of certain cells. This investigator noted that certain organisms have a particular breakdown constant (K) or mechanical fragility. The following table

shows the relative mechanical fragility of certain cells together with their approximate diameters (Ackerman, 1952b).

Cell	Mechanical Fragility K/K Human RBC	Approximate Average Diameter (Microns)
P. aurelia	16	80
P. caudatum	4	150
Amphiuma tridactylu RBC	3	50
T. foetus	2	12
Human RBC	1	6
Rabbit sperm	0.7	5
Amoeba proteus	0.4	200
T-2 bacteriophage	0.2	0.01
E. coli	0.15	1
Staph. Albus	0.07	1
E. coli B	0.01	1
Baker's yeast	0.0003	5

From this table it appears larger cells exhibit a greater mechanical fragility to ultrasound than smaller cells. Since plasmodia can resist higher pressures than erythrocytes (D'Antonio et al., 1966); and since *P. berghei* is smaller than an erythrocyte, then theoretically it is possible to rupture the erythrocyte with sonic energy releasing unaltered plasmodia.

Release of Plasmodia from Erythrocytes Using Ultrasonic Energy

Verain and Verain (1956) first reported the liberation of malaria parasites from erythrocytes using ultrasonic energy. These

investigators ultrasonically irradiated rodent erythrocytes infected with Plasmodium berghei with an intensity of 4 watts/cm^2 and frequency of 1 MHz. They reported the liberation of Plasmodium berghei which appeared to be unaltered, and some parasites retained their infectivity. On longer exposures to ultrasonic energy, Verain and Verain (1956) found morphologic deformations of the freed parasites. Furthermore, these investigators found that the deformed parasites lost their infectivity.

Kreier et al. (1965) reported incomplete separation of Plasmodium gallinaceum from host cell stroma when the infected erythrocytes were treated with a formalin-saponin solution. But these investigators found that exposure of the formalin-fixed saponized infected erythrocytes to ultrasonic energy liberated plasmodia which were morphologically unaltered. Weiss (1967) exposed washed preparations of Plasmodium gallinaceum-infected erythrocytes to ultrasonic energy and reported destruction of the erythrocytes with concomitant release of the plasmodia parasites. Weiss (1967) also reported the free parasites appeared to be morphologically unaltered.

Rutledge and Ward (1967) have also reported the release of malaria parasites by exposing infected erythrocytes to sonic energy. These investigators showed that when a batch of Plasmodium gallinaceum-infected erythrocytes is exposed to sonic energy, parasites are released from their host cells; however, on continued exposure to ultrasound, the free parasites disintegrate. Prior (1968) also showed that during batch sonication, parasites are freed and then undergo morphological deformation and destruction. It is apparent from these results that no

appreciable quantity of free parasites can be obtained utilizing batch sonication. As a consequence of these observations, most investigators have been led to believe that ultrasound cannot be used to produce large quantities of parasitic antigens.

The primary purpose of this study was to obtain quantities of morphologically intact parasites by ultrasonic treatment of parasitized erythrocytes. This objective was accomplished by utilizing a continuous-flow system of sonication in which the freed parasites were promptly removed from the disruptive effects of the cavitating ultrasonic field.

STUDIES OF ULTRASONICALLY-FREED PARASITES

Examination of Sonically-Freed Parasites by Electron Microscopy

Examination of the free parasite preparation by low power thin-section electron microscopy is necessary 1) to determine if the parasites are truly free of their host cell membranes and 2) to determine if any parasite damage has occurred and, if so, to what extent.

Cook et al. (1969) showed by thin-section electron microscopy that saponin lysed malaria-infected red cells do not yield free malaria parasites as was originally asserted by Christophers and Fulton (1939). Cook et al. (1969) point out that erythrocyte membranes, per se, cannot be resolved by light microscopy unless large sheets of membrane are viewed en face. Consequently, the electron microscope is necessary if these "invisible" host cell membranes are to be seen. Furthermore, structual damage to the parasites can only be ascertained using the electron microscope.

To this reviewer's knowledge, there is not as yet any literature in addition to Prior (1968) on the morphological examination by electron microscopy of plasmodia liberated from erythrocytes by ultrasonic energy. As mentioned above, low-power electron microscopy must be used to substantiate the statement that a preparation contains "free" malaria parasites. Cook et al. (1969) believe that none of the methods purporting to yield "free" parasites do, in fact, yield parasites free of host cell membranes. Accordingly, controversy still surrounds the techniques used to produce plasmodial antigens (Cook and Aikawa, 1971; and D'Antonio and Silverman, 1971). However, Trigg et al. (1970) recently reported producing preparations of free malaria parasites by nitrogen cavitation. These investigators reported most, but not all, of the malaria parasites were free of host cell membranes as determined by thin-section microscopy. However, these investigators reported parasite damage had occurred to some extent using this procedure. Martin et al. (1971) have also reported the complete separation of malaria parasites from host erythrocytes by lysing the erythrocytes with ammonium chloride.

Immunological Studies Using Free Parasites as Test Antigens

An immunological test is no better than the antigen on which it depends (Fife, 1970). Malaria-infected animals as well as animals with other diseases commonly have in their serum antibodies to modified erythrocyte membrane components (Kreier et al., 1966; Kreier, 1969). These antibodies may react with host membrane components when antigens are made by current methods. Accordingly, the results of the test may be obscured.

Methods to produce malarial antigens which are used in serological tests vary from laboratory to laboratory and also vary with the type of test performed. For example, in complement-fixation tests, Pautrizel and Nien (1953) used infected blood which was lysed in distilled water and then treated with trypsin as the test antigen. D'Antonio et al. (1966a) used antigens in complement-fixation tests prepared by the French pressure cell. Stein and Desowitz (1964) used infected blood hemolyzed in distilled water and then homogenized as an antigen preparation in a hemagglutination test. Obviously, results obtained in one laboratory often cannot be compared with the results obtained in another laboratory. In addition, contradictory results are sometimes published in the literature. Brown (1969) has written an excellent review on the immunological aspects of malaria infections. In this review, Brown includes a review of malarial antigens and their collection as well as a review on the humoral factors in malarial immunity.

In conclusion, an antigen preparation free of host cell contamination would certainly be of value in serological and biochemical studies. For example, a solubilized free plasmodial preparation used properly in the standard complement-fixation test should increase the sensitivity and specificity of the test, and thereby inhance confidence in the test results.

CHAPTER II

M A T E R I A L S A N D M E T H O D S

CONTINUOUS-FLOW SONICATION SYSTEM

Description of System

The continuous-flow sonication system was designed and assembled in the laboratories of the Department of Microbiology and the Department of Welding Engineering of The Ohio State University. The system consisted of a pump, sonic generator, and continuous-flow sonication chamber.

The pump used was a Harvard Rotary Peristaltic Pump Model 1225 (Harvard Apparatus Co., Inc., Millis, Massachusetts). This pump utilized standard 1/4 x 1/8" Tygon tubing for transmission of the specimen. Flow rate of the specimen was variable, thus making this pump extremely versatile. Maximum flow rate available was 29.6 ml/min.

The sonic generator used was a commercially available Bronwill Biosonik Model BP 1 generator (Bronwill Scientific, Rochester, New York). This generator was chosen because it was not expensive and because similar generators are quite common in most laboratories.

The model used in this study contained a sonic-power control and a tuning control. The power dial is divided into 100 increments which represent relative acoustic intensity or power. The tuning control changed the excitation frequency slightly so as to compensate for

variations in load characteristics, etc. The frequency of the ultrasonic waves used was 20,000 Hertz (cycles per second).

The radio frequency (rf) power produced by the electronic generator was converted at the same frequency into mechanical (acoustic) power by a magnetostrictive transducer. The transducer was equipped with a standard 3/8" probe tip.

Continuous-Flow Sonication Chamber

Several of the chambers tested were designed so that the transducer probe tip was in indirect contact with the biological specimen. These chambers were constructed of glass, Tygon tubing, and brass shim of various thicknesses. Coupling fluids tested were water and glycerin.

In none of these chambers was sufficient cell breakage induced at the power and flow rates possible to warrant further study. For example, a chamber constructed of S-3 0.003" thick brass shim with the transducer probe tip 1/4" above the chamber yielded about 5% cell breakage with maximum power and a flow rate of only 2.0 ml/min. Lowering the transducer probe tip closer to the chamber increased the cell breakage to about 40% with maximum power. This high power setting induced heating. No appreciable differences in cell breakage were observed between the water and glycerin coupling fluids.

Next chambers were designed so that the specimen was in direct contact with the transducer probe tip. A standard commercial continuous-flow sonication chamber (Bronwill Scientific, Rochester, New York) was modified by reducing the void volume of the sonication chamber. This modification was made to reduce eddying in the specimen and to equalize

exposure to all cells in the specimen, thereby reducing debris formation.

Good results were obtained using this modified chamber. Power dial settings as low as 30 yielded approximately 80 percent red cell breakage with a flow rate of 29.6 ml/min. Heating of the specimen was slight as was debris formation.

After reproducible results were obtained with the modified chamber, a new continuous-flow sonication chamber was constructed using 316 stainless steel which incorporated the best features of the modified chamber and included some additional variations which hopefully would yield better results. The cooling jacket was eliminated in the new chamber as heating was not a problem. Fig. 2 is a blue-print drawing of the new continuous-flow sonication chamber.

Fig. 3 shows the chamber placed on the transducer. The only place of direct contact between the chamber and the transducer is the point where curvature begins on the probe. At this point, an "O" ring seals the chamber and secures it to the probe tip. This design feature allows the transducer to vibrate freely. The entrance to the chamber accommodates the standard 1/4" x 1/8" Tygon tubing from the peristaltic pump. Void volume of the actual sonication chamber is 0.0138 cubic inches or 0.2 cubic centimeters. At a flow rate of 29.6 ml/min., each red cell receives approximately 4,000 cycles of ultrasonic energy.

A TYPICAL RUN

Preparation of Specimen for Sonication

Charles River inbred rats were the experimental animals used in

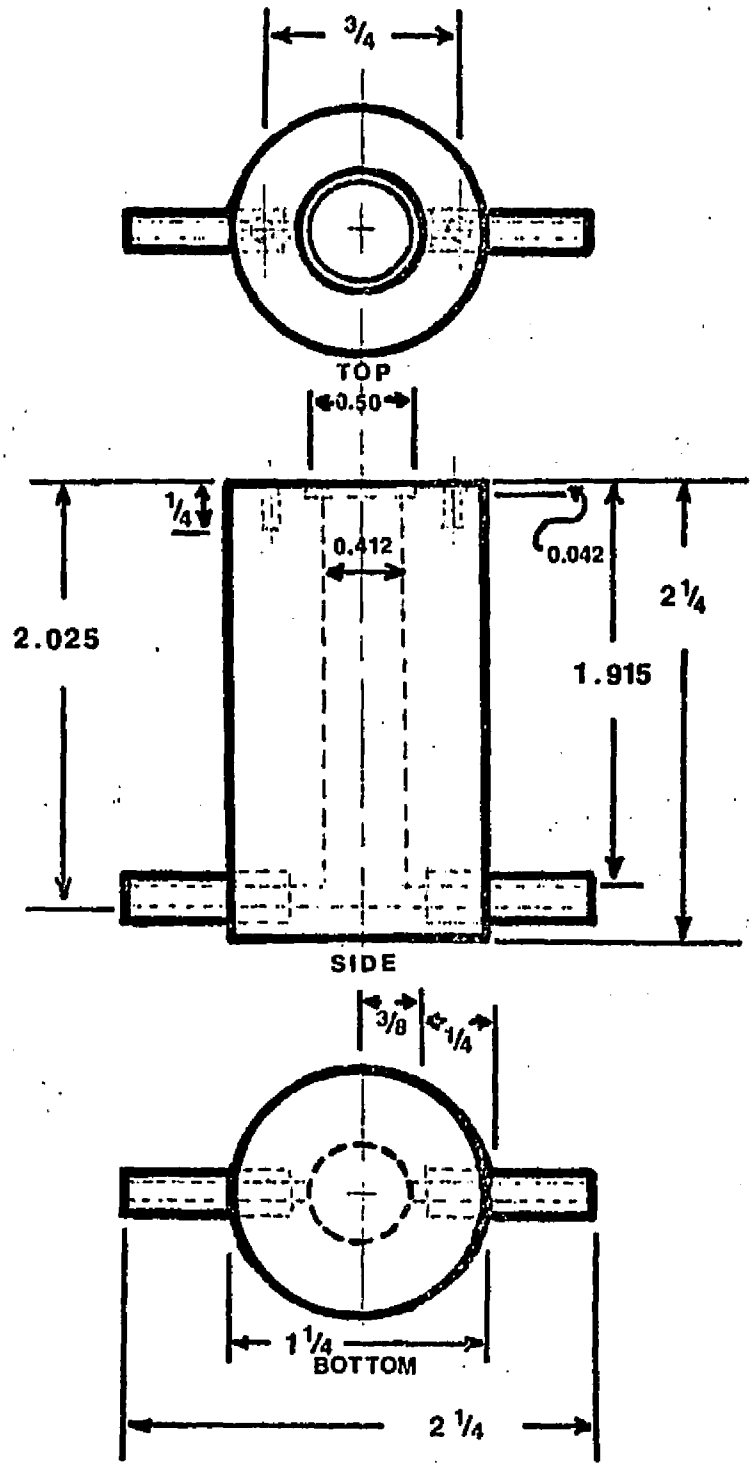


Fig. 2. Continuous-flow sonication chamber. (All measurements are in inches.)

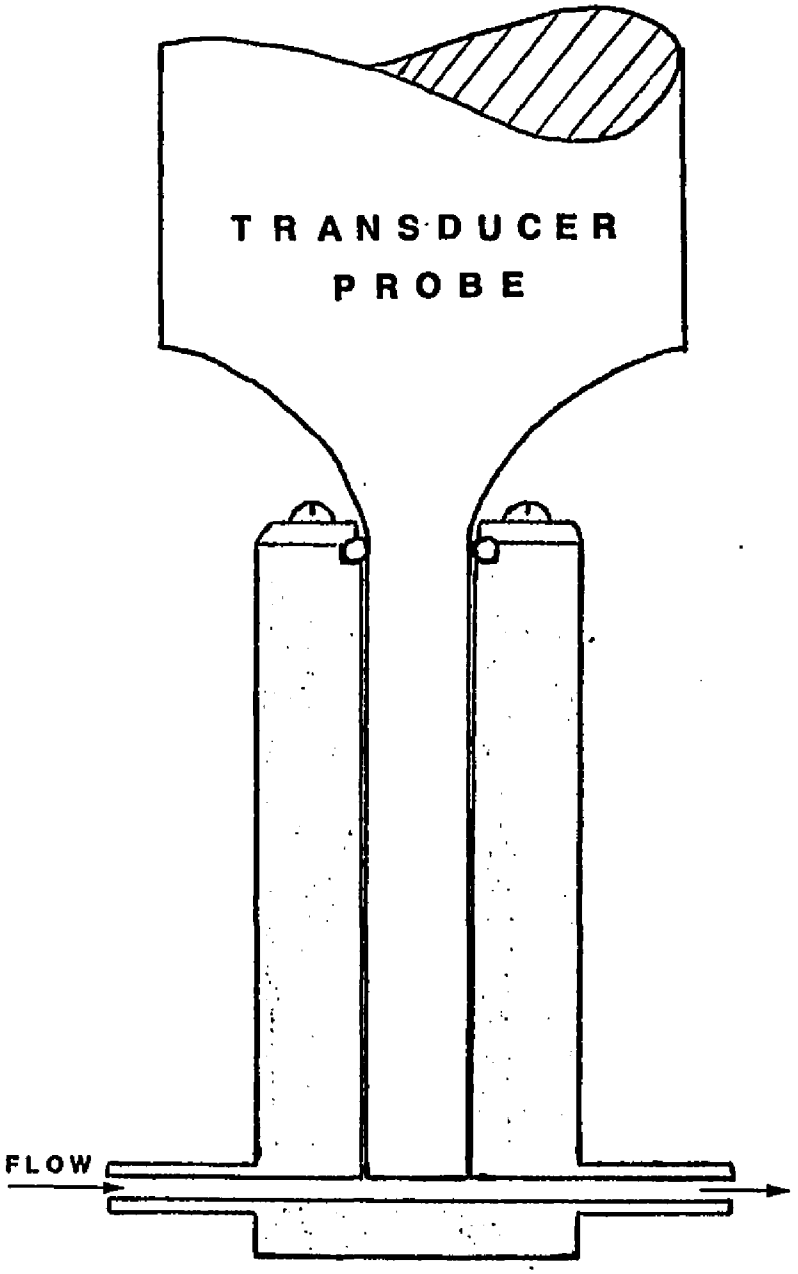


Fig. 3. Diagram of continuous-flow sonication chamber assembled and affixed to the transducer probe.

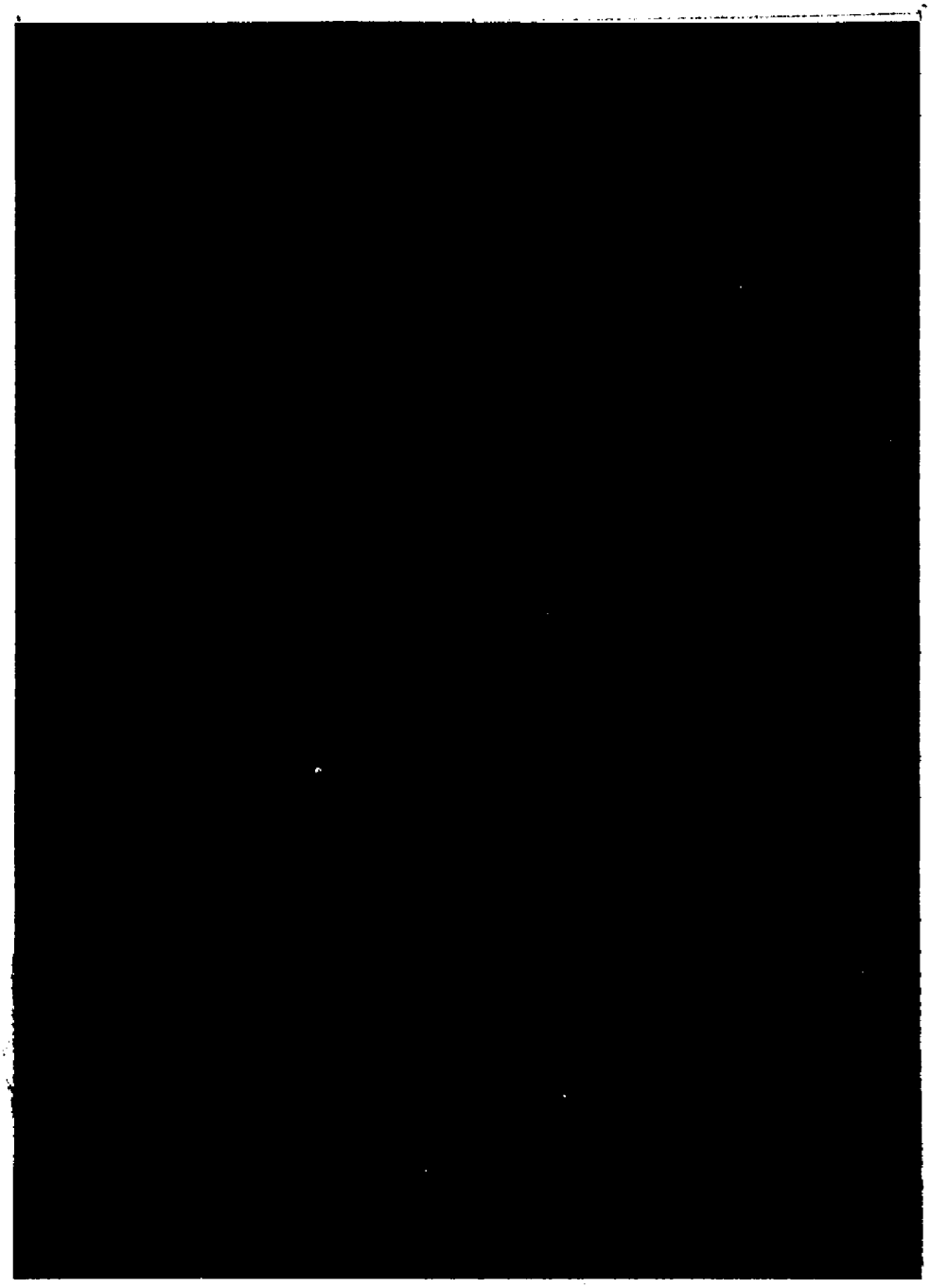
this study although occasionally random bred albino rats were used. These animals ranged from 5 to 10 weeks of age and were caged at The Ohio State University College of Biological Sciences' animal facilities. The rats were maintained on a diet of Purina Lab Chow, and water was given ad libidum.

The Plasmodium berghei strain used in this study was originally obtained through the courtesy of Dr. Herbert Cox, New York University and was maintained in the laboratory by injecting approximately 5×10^7 to 1×10^8 parasitized erythrocytes intraperitoneally into susceptible rats. The susceptible rats developed a parasitemia within two to three days. This pre-patent period varied and was dependent upon dose and age of animal. The susceptible rats developed a parasitemia which on occasion was as high as 95 percent, again depending on the age of the animal. The usual time required for peak parasitemia was about 8-10 days after injection of infected blood. The strain of Plasmodium berghei produced an asynchronous patent infection in the rats.

Infected blood which had greater than 50 percent parasitemia was used throughout in this study. A typical population of rat erythrocytes highly parasitized with Plasmodium berghei is shown in Figure 4. Such blood was obtained from the rats as follows:

1) The donor rats were anesthetized with ether;
2) A mid-line laporatomy was made to expose the heart using a pair of surgical scissors;
3) A 21 Gauge 1 1/2" long needle affixed to a 10 ml syringe was inserted into the right auricle and blood was withdrawn. EDTA was used as the anticoagulent (see appendix).

Fig. 4. Photomicrograph of Giemsa-stained rat erythrocytes showing a high parasitemia of Plasmodium berghei. X 2,000.



Average yield for a 200 gram rat was about 8 ml whole blood. The blood from 2 or 3 rats was pooled and centrifuged in the cold at 500 x G for 15 min. Following centrifugation, the supernatant plasma and buffy leukocyte coat were aspirated. The erythrocytes were subsequently washed twice (500 x G for 15 min.) in Alsever's solution pH 7.2 (see appendix).

Leukocytes not removed by aspiration were routinely removed by filtration of the washed erythrocytes through columns of packed powdered filter paper. The procedure used was similar to those described by Cook et al., 1969; and Fulton and Grant, 1956. Whatman Column Chromedia CF-11 Fibrous Cellulose Powder was tightly packed in a 10 ml syringe over a plug of glass wool. Approximately 2 cubic centimeters of powdered filter paper were used in each syringe. The washed blood was poured into the inverted syringe, and the plunger was replaced. The blood was then slowly forced through the filter paper column by exerting a slight pressure on the plunger. Time to filter 10 ml of washed blood was about one minute. Examination of Giemsa-stained smears before and after filtration showed that virtually all remaining leukocytes not removed in the buffy coat were removed by the passage through filter paper powder.

The hematocrit value expressing the percent of red blood cells in the washed blood was determined by filling a standard heparinized microhematocrit capillary tube (Clay Adams, Inc., New York, New York) approximately 3/4 full with the washed blood. The red end of the hematocrit tube was sealed with plastic clay (Seal-Ease, Clay Adams, Inc., New York,

New York), placed in an International Micro-Capillary Centrifuge Model MB (International Equipment Co., Needham Hts., Massachusetts) and centrifuged at 12,000 x G for five minutes. The hematocrit value was read on an International Micro-Capillary Reader (International Equipment Co., Needham Hts., Massachusetts). The washed erythrocytes were then made up to a concentration of 10.0 ± 0.5 percent by volume as judged by this high speed hematocrit determination.

A Giemsa smear was then made of the specimen, and it was from this smear that the percent parasitemia of the test blood to be sonicated was determined. Percent parasitemia was determined by counting the number of infected erythrocytes as a function of total number of erythrocytes in a microscopic field. At least 500 erythrocytes were counted per smear, using a standard bright field microscope. In addition to the determination of percent parasitemia, an actual cell count was made using a standard Spencer Bright Line Hemocytometer. The counting procedure was the same as that described by Schalm (1961). Counts were expressed as the number of cells per cubic millimeter.

The properly-labeled specimen was then stored in the cold (4°C) until needed for sonication. All blood was sonicated within 24 hours after preparation.

Determination of Flow Rate and Sonic Intensity

Prior to sonication of infected blood, normal rat blood prepared in exactly the same manner as described above was used to evaluate the continuous-flow sonication system. Once proper parameters were established, infected blood was used exclusively.

The two most important parameters in the continuous-flow sonication system were the flow rate of the specimen and the sonic intensity within the chamber. The flow rate was set at 29.6 ml/min., the maximum possible with our pump to minimize the duration of direct exposure to the acoustic energy. With flow rate held constant, the relationship of acoustic intensity to red cell breakage was determined. As normal rat erythrocytes passed through the chamber, the power dial was advanced, and samples of the sonicated specimen were collected for subsequent determination of degree of hemolysis. This step was repeated for each power dial setting.

The percent hemolysis was determined by counting the number of red cells in the sonicated samples using a standard hemocytometer. The relationship used was:

$$\% \text{ hemolysis} = \frac{\text{number of red cells/mm}^3 \text{ after sonication}}{\text{number of red cells/mm}^3 \text{ before sonication}} \times 100$$

The percent hemolysis was then plotted against the power dial setting (relative acoustic intensity). Typical results are shown in Fig. 5. It can be seen that beyond 85 percent hemolysis, little increase in red cell breakage occurs with increasing acoustic power settings. In fact, microscopic examination of specimens sonicated at power levels which caused over 85% hemolysis showed significant debris formation. Consequently, the proper power level to sonicate specimens flowing at a rate of 29.6 ml/min. was determined to be that setting which yielded approximately 80 to 85 percent red cell breakage. Therefore, by using percent hemolysis as a criteria for power level settings, variables which affect

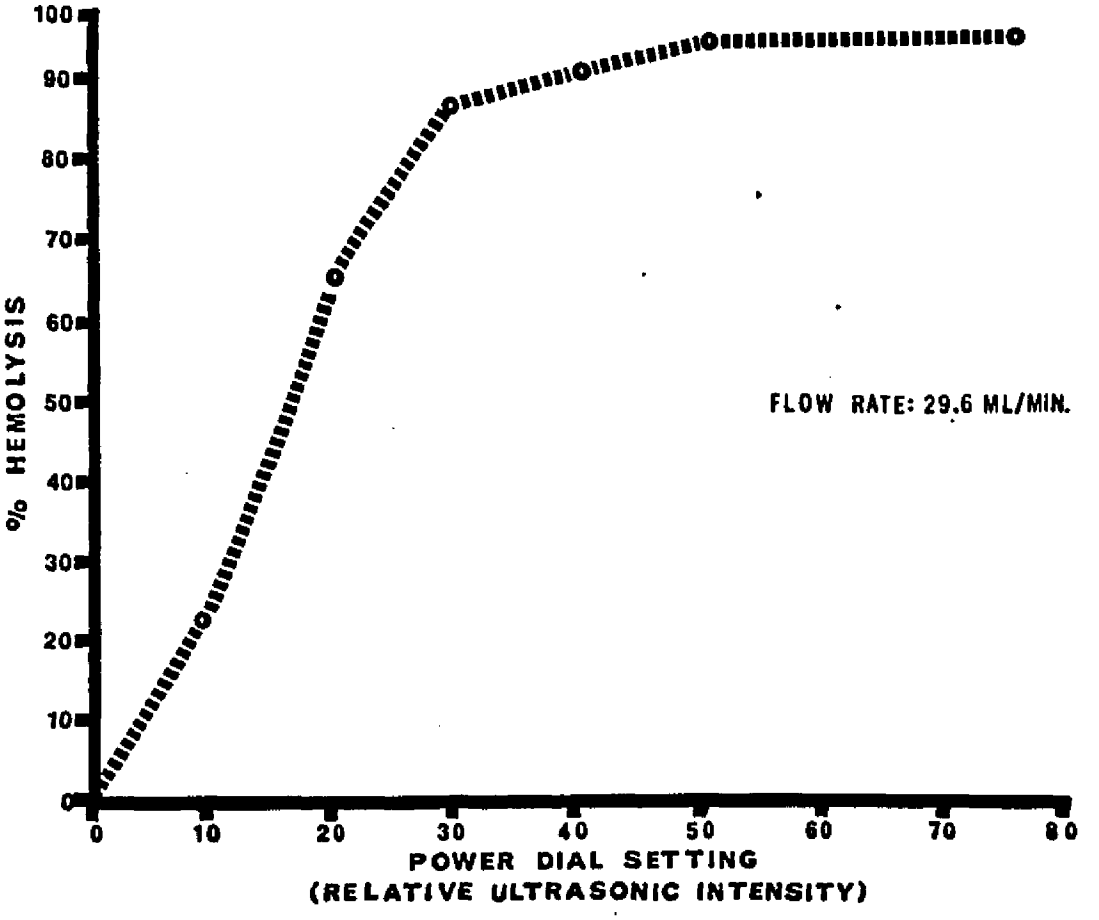


Fig. 5. Graph showing the rapid increase of hemolysis followed by a sharp reduction in the rate of increase of hemolysis with increasing ultrasonic intensity.

acoustic cavitation such as gas content of the liquid were eliminated, and reproducible results were obtained.

Sonication of Specimen

Once the performance of the system had been evaluated, erythrocytes from rats infected with Plasmodium berghei were sonicated. Erythrocytes which had been previously washed, filtered to remove leukocytes, and adjusted to a hematocrit of 10 percent were thoroughly mixed prior to sonication. The continuous-flow sonication chamber was assembled and affixed to the transducer probe. The Tygon tubing from the peristaltic pump was attached to the chamber, and the generator was turned on and allowed to warm up. Following these preliminary setup procedures, the open end of the Tygon tube leading to the peristaltic pump was inserted into the flask containing the specimen. The peristaltic pump was turned on, and the power level on the generator was adjusted to a setting which produced approximately 80 to 85 percent hemolysis. As the blood passed through the chamber, a characteristic "buzzing" or "hissing" sound was heard which indicated proper operation (resonance) of the system. On occasion, this sound would cease; and then the tuning control on the generator was adjusted to return the system to resonance. A distinctive color change of the blood was also observed which indicated proper operation of the system. The bright red of the specimen changed to a darker-colored red after sonication. Time to sonicate a 50 ml sample was just under 2 minutes.

Following sonication, a hematocrit determination as well as an actual cell count were made. Examination of the sonicated specimen by

both light microscopy of Giemsa-stained specimens and phase-contrast microscopy showed many free parasites. The free parasites appeared morphologically intact. In addition to the free plasmodia, whole erythrocytes, ghost erythrocytes, and some debris were observed. Separation of these free plasmodia was the next step.

HARVESTING THE FREE PARASITES BY DIFFERENTIAL CENTRIFUGATION

Determination of Sedimentation Rate of Rat Erythrocytes

The separation procedure used was differential centrifugation.

The sedimentation rate of rat erythrocytes was determined by the following technique:

1) Standard microhematocrit capillary tubes were filled approximately 3/4 full with the washed erythrocytes prepared as a 10 percent suspension and one end sealed with plastic clay.
2) The tubes were placed in a vertical position and stored in the cold (4°C).
3) Each hour, the distance the red cells had sedimented from the top of the fluid was measured.
4) The total distance the red cells moved was then divided by the total time in hours.

The sedimentation rate of the washed rat erythrocytes was calculated to be 1.5 mm/hr. at 1 x G force.

The above sedimentation value was converted into mm/min. and multiplied by 250 x G force. The procedure was repeated for various distances and times, and the results are shown in Fig. 6. By this procedure,

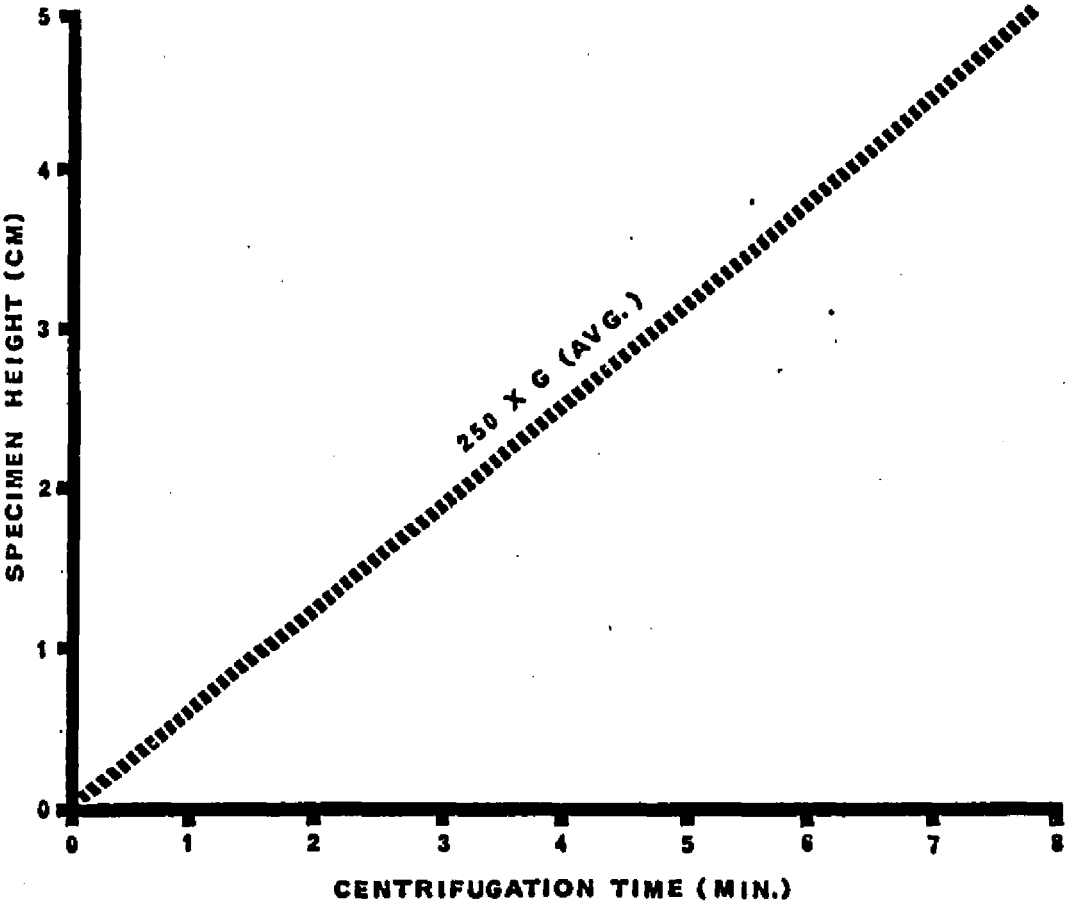


Fig. 6. Sedimentation rate curve of washed rat erythrocytes.

the spin time necessary to sediment all the erythrocytes at a 250 x G force was determined.

Centrifugation Procedure

The actual mechanics of centrifugation were very important in achieving maximum yield of parasites relatively free of host cell contaminants. The two most important factors for achieving this were 1) the use of a swinging-bucket type centrifuge and 2) accurate calibration of the centrifuge.

The centrifuge used in this study to separate parasites from the sonicated specimens was the International Model CF Centrifuge equipped with a #215 swinging bucket head (International Equipment co., Needham Hts., Massachusetts). Centrifuge tubes ranging in size from large wide-mouthed types to small 12-ml tubes could be accommodated by this head using the appropriate rubber inserts. Speed was variable with maximum speed about 2500 rpm.

The precise number of rpm's achieved at the various dial settings was determined using a General Radio Type No. 631-B Strobotac Stroboscope (General Radio Co., Cambridge, Massachusetts). The gravity forces produced at the various speeds used were determined using the nomograph supplied by the International Equipment Co. In addition to determining the speed achieved, the times required to reach those speeds were determined. Acceleration rates vary among centrifuges; and for the one used in this study, the time to maximum acceleration averaged one minute for all speeds tested. This one minute was added to all calculated centrifugation times.

Following sonication, the specimen was divided into a number of equal aliquots and placed in centrifuge tubes. The height of the specimen in the tubes was measured, and the specimens were then centrifuged at 250 x G (avg.) for the time determined from the sedimentation rate curve (Fig. 6). Immediately following this initial spin, the red supernatant fluids were decanted carefully and placed in other centrifuge tubes. Examination of the supernatant and pellet by light microscopy following Giemsa staining and by phase-contrast microscopy revealed that the supernatant contained large numbers of free parasites of various sizes together with some ghost cells and very little debris. The pellet contained virtually all intact rat erythrocytes with some free parasites. These intact rat erythrocytes found in the pellets after the first spin were those which were not lysed by the acoustic cavitation. The pellets were discarded.

The next centrifugation was carried out at a gravity force sufficient to sediment the free parasites and leave in the supernatant the ghost cells, host membranes, and debris. The actual sedimentation value for the free parasites could not be determined as 1) they could not be seen macroscopically as could the red cells and 2) they sedimented at various rates because they had a variety of sizes. Consequently, test samples were centrifuged (600 x G) for various times, and the supernatant and pellet were examined microscopically. Results showed that centrifuging the supernatant at 600 x G for 3 minutes for each centimeter of height produced the maximum number of free parasites with minimum contamination. Examination of this supernatant and pellet by

both light microscopy of Giemsa-stained smears and phase-contrast microscopy revealed that the supernatant contained few free parasites (the smaller ones being predominant in the field), many ghost cells, host cell membrane components, some debris while the pellet was predominately free parasites of all sizes, a few ghost cells and very little debris. The red supernatant was discarded, and the free-parasite pellet was then washed twice in 5 ml Alsever's pH 7.2 (600 x G for 15 min.). The centrifuge tube used was a standard Kimax 12 ml centrifuge tube.

The physical appearance of the pellet clearly showed that the different sizes of parasites had various sedimentation rates. The bottom of the pellet was a dark brown; above this was a layer which was light brown; and above this was a gray-colored layer. Occasionally, a small, white, fluffy layer was observed atop the free-parasite pellet. Examination of this layer showed it consisted chiefly of ghost cells; consequently, this layer was routinely aspirated off. The free-parasite pellet was rather smooth in consistency and dispersed quite readily in the washing fluids. It was this pellet, after the washings, that was used in the various morphological and immunological studies.

Enumeration of Free Parasite Yield

Determining the yield of free parasites was difficult as 1) the free parasites have a tendancy to clump and 2) they vary in size, the smallest being less than a micrometer in diameter. Techniques using such devices as the Cell-O-Scope Counter and some techniques using the standard hemocytometer were tried, but they gave inconsistent results.

Subsequently, two procedures were tried and evaluated and gave satisfactory results.

The first procedure involved the use of the standard hemocytometer counting chamber. Since the larger parasites could be seen readily, counts of these larger forms were made before and after centrifugation. Results showed approximately 90 percent recovery of these parasites. To make the smaller parasites more easily visible, Kolmer's Counting Fluid (see appendix) was used as the diluent in the counting process. Actual counts of a suspension of free parasites (5% packed cell volume) indicated a free parasite count of approximately 9×10^9 free parasites per cubic centimeter. A mathematical analysis assuming an average of three parasites per infected cell and an 85 percent red cell breakage in the sample for which this preparation was obtained showed that approximately 10×10^9 parasites were present if the yield were 100%. In addition, the volume these parasites would occupy was calculated, and this figure agreed quite well with the actual packed free-parasite volume obtained.

Another procedure was also tried to determine the number of free parasites recovered by the differential centrifugation procedure. This procedure consisted of counting a solution of 1.7 µm latex balls with the hemocytometer. (The refractive properties of the latex balls made them easy to count.) A 0.05 ml quantity of this standard solution was pipetted, using a microtiter pipet, into an equal volume of the free parasite preparation. The solution was thoroughly mixed, and a drop was placed on a slide. The solution was examined under a phase contrast microscope (high power), and the ratio of latex balls to free parasites

was determined. Again the results correlated quite well with those obtained above. Depending on the development stage of the parasite at the time of blood collection, a 1 ml solution of a 5% packed volume of free parasites contained 9×10^9 to 4×10^{10} free parasites. These figures indicated approximately 80 to 90 percent recovery of those parasites freed by the cavitating ultrasonic field.

PREPARATION OF SPECIMEN FOR MICROSCOPIC EXAMINATION

Introduction

In order to observe the morphological appearance of the freed parasites in the pellet, the following microscopic techniques were used:

1) bright-field microscopic examination of Giemsa-stained preparations
2) phase-contrast microscopic examination of unstained preparations
3) interference-contrast microscopic examination of unstained preparations
4) fluorescent microscopic examination of acridine-orange-stained preparations
5) electron microscopic examination of ultra-thin sections
6) electron microscopic examination of carbon replicas

The free-parasite pellet was processed for these various microscopic techniques by the methods described below.

Giemsa Stain

Thin films of the free-parasite pellet (and the various supernatants and pellets described earlier) were prepared on pre-cleaned

microslides (Clay Adams, Inc., New York). The films were then allowed to air dry and were subsequently fixed for 3 minutes in absolute methyl alcohol. Following fixation, the films were air dried, placed on a staining rack, and flooded with dilute Giemsa stain for a period of 20 minutes. The Giemsa-stain solution was prepared by adding one ml of the stock solution (The Ohio State University Reagents Laboratory) to 25 ml of phosphate buffer pH 7.3 (see appendix). After staining, the films were rinsed with distilled water and air dried. Fresh dilutions of the stain were prepared before each use.

Each film was subsequently examined, and photomicrographs were taken of representative fields. A Carl Zeiss Standard WL Microscope (#4376162) with phase-contrast condenser III-Z (W. H. Kessel and Company, Scientific Instruments, Chicago, Illinois) and equipped with a Honeywell Pentax Camera, Model H3 (Asahi Optical Co., Japan) was used. The camera was attached to the microscope by an Asahi microscope adapter. Kodachrome II Type A 35 mm professional film was used where colored micrographs were required. Fine-grain Panatomic X film was used for all black and white micrographs (Eastman Kodak Co., Rochester, New York). The 100X, N. A. 1.30 PH-3 oil immersion objective lens was used, and the condenser was set on "J" for bright-field microscopy.

To insure accurate size measurements, a Bausch and Lomb stage micrometer (Bausch and Lomb Optical Co., Rochester, New York) was photographed at those magnifications used when photographing the biological specimens. The stage micrometer scale was ruled to 0.01 mm. The photographed scale was the basis for all cellular measurements made using the

Carl Zeiss Standard WL Microscope. These included the Giemsa, phase-contrast, interference-contrast, and acridine-orange fluorescent micrographs.

Phase-Contrast Microscopy

Phase-contrast microscopy proved to be extremely important during the developmental stages of the continuous-flow sonication procedures. Examination of the supernatants and pellets during the differential-centrifugation procedure by phase-contrast microscopy also proved to be a quick and reliable method to evaluate the effectiveness of the centrifugation procedure. Many erythrocyte membrane components could be detected by phase-contrast microscopy.

A drop of the specimen to be examined was placed on a pre-cleaned slide using a pasteur capillary pipet. A microglass cover slip (22 mm square) was then placed on the specimen, and the specimen was then examined by the phase-contrast microscope. For subsequent photomicrographs, the particular field was left untouched for several minutes so the specimen would "settle" and cease to move. The equipment used was the same as that described in the Giemsa-stain section.

Interference-Contrast Microscopy

The procedure used for interference-contrast microscopy was the same as that described for phase-contrast microscopy, except that the equipment used was the standard Carl Zeiss WL microscope equipped with an interference-contrast condenser. Photomicrographs were taken of representative fields of the free-parasite pellet.

Acridine-Orange Fluorescent Microscopy

The procedure used for the acridine orange study of the free-parasite pellet was similar to the procedure described by LeCover and Warner (1958). Thin films of the free-parasite pellet were prepared on pre-cleaned microslides and fixed for three minutes in absolute methyl alcohol. Following fixation, the slides were air dried and then placed in a Copeland jar filled with acridine orange fluorochrome diluted 1:20,000 (see appendix). The slides were allowed to stain for exactly 2 1/2 minutes after which they were washed for 1/2 minute in acridine-orange buffer pH 9 (see appendix). The slides were then examined with the fluorescent microscope.

The microscope used was the Carl Zeiss Standard WL Microscope with a Carl Zeiss ultraviolet attachment (#4655101) using an Osran mercury super pressure lamp HBO 200 W (Macbeth Corp., Newburgh, New York). Emitter filters I and II and barrier filters 50 and 44 were used. The camera and film used to take the photomicrographs of the acridine-orange preparation were the same as those described in the "Giemsa-stain" section.

Thin-section Electron Microscopy

For study by electron microscopy, the free-parasite pellets were processed by the method described by Cook et al. (1969). Basically, the procedure was as follows:

1) The samples were fixed overnight (4°C) in 1.25 percent glutaraldehyde with 1/15 molar phosphate buffer and 4 percent sucrose, pH 7.3 (see appendix).

2) The fixed samples were washed thoroughly in 1/15 molar phosphate buffer pH 7.3 containing 4 percent sucrose, then postfixed in 1 percent osmium tetroxide in 1/15 M phosphate buffer pH 7.3 for one hour at 4°C (see appendix).
3) The samples were dehydrated in an ascending ethanol series to 100 percent ethanol, then propylene oxide was added twice to remove the alcohol.
4) The samples were subsequently embedded in Epon 812 (Luft, 1961) (see appendix).

Certain procedures were found necessary to insure proper impregnation of the monomer into the free parasites. These included the overnight storage of the samples in the cold (4°C) with the complete monomer after which fresh monomer was added prior to polymerization. The best polymerization schedule was found to be storage overnight at 35°C, storage the next day at 45°C, and the overnight storage at 60°C.

Following polymerization, the specimens were sectioned with a Reichert "OmU 2" Ultramicrotome equipped with a glass knife. The glass knives were made using the LKB Knife Maker Model 7800B (LKB Instruments, Inc., Rockville, Maryland). The collecting trough attached to the glass knife was filled with a 10 percent acetone solution. The silver-colored serial sections cut from the Epon blocks were mounted on uncoated 400 mesh copper E. M. specimen grids (3 mm O. D.) (Mason and Morton Limited, Middlesex, England).

After the specimens were properly mounted on the EM grids, they were poststained with uranyl acetate and lead citrate. Specimens were placed in a filtered, saturated solution of uranyl acetate in 50 percent

ethanol for 30 minutes in the dark. They were then rinsed in 50 percent ethanol, followed by distilled water. The grids were then placed in lead citrate for 15 minutes (Reynolds, 1963). This was followed by a distilled water rinse and a 0.02N NaOH rinse. The grids were then dipped in 100 percent ethanol and air dried. The grids were never permitted to dry during the staining procedure.

The specimens were then examined with the Zeiss EM 9S electron microscope. Photographs were taken of representative fields.

Carbon Replica Electron Microscopy

The carbon replica technique used was the one described by Bradley and Williams (1957).

The free parasites were fixed overnight at 4°C in 1.25 percent glutaraldehyde with 1/15 molar phosphate buffer and 4 percent sucrose pH 7.3. The fixed free parasites were then washed thoroughly in distilled water, and a drop of the preparation was placed on each of several formvar-coated 300 mesh copper grids. When dry, the grids were shadowed with germanium and coated with carbon. The formvar coating was removed by washing the grids several times with chloroform. Then the underlying organic material was oxidized by floating the grids for 2 1/2 minutes on a freshly prepared solution of 1.5 gm of potassium dichromate, 1.5 gm of potassium permanganate, and 15 ml of concentrated sulfuric acid. The grids were subsequently washed in distilled water and air dried. The carbon replicas of the free plasmodia were examined with the Zeiss EM 9S electron microscope, and photographs were taken of representative fields.

PREPARATION OF SPECIMENS FOR COMPLEMENT FIXATION TESTS

Free-Parasite Antigen

One volume of packed free parasites was suspended in 19 volumes of Triethanolamine-buffered salt (TBS) solution (see appendix). This yielded a 5 percent by volume suspension of the free parasites. The actual volume of the free parasites was determined directly from the graduated markings on the 12 ml Kimax centrifuge tubes after centrifuging at 600 x G for 15 minutes. The primary reason for adjusting the free-parasite concentration to a standard concentration was to establish a basis for all subsequent dilutions. Consequently, all dilutions mentioned in the complement-fixation test procedure are expressed as dilutions of the 5 percent packed free-parasite suspension.

Following adjustment of parasite concentration, the parasites were lysed by repeated cycles of freezing and thawing. This was accomplished by placing the specimen in a CO_2-acetone bath until frozen, then into a 56°C water bath until thawed for three complete cycles. The liquid was light brown in color, and traces of dark brown membraneous material could be seen floating on the liquid. The liquid was then exposed to a few short bursts of ultrasonic energy. The actual procedure consisted of placing the transducer probe tip into the liquid and turning the power dial setting to 50 for 2 seconds. The resulting light-brown solution appeared clear and was used, when diluted, in the complement-fixation tests.

Lysed Parasitized Erythrocyte Antigen

For control purposes, a lysed parasitized erythrocyte antigen was prepared employing hypotonic lysis. The method used was similar to that described by Dulaney (1944) who used hypotonic lysis for release of plasmodia from the erythrocytes. However, it has since been shown that hypotonic lysis does not free the plasmodia from their host cell membranes.

Infected rat blood was washed three times in Alsever's solution pH 7.2. The packed erythrocytes were lysed in 10 volumes of double distilled water and immediately centrifuged in the cold (4°C) at 1500 x G for 15 minutes. The supernatant hemoglobin solution was removed by aspiration, and the sediment was then washed three times in TBS pH 7.3. Following each wash, the fluffy brown layer consisting primarily of red-cell stromata was aspirated from the packed parasites. The parasite pellet was then resuspended in 19 volumes of TBS pH 7.3. This 5 percent packed parasite solution then was solublized by the same procedure as described in the free-parasite section.

Rat-Erythrocyte Membrane Antigen

Membranes of erythrocytes of normal rats were also employed as test antigens. Such antigens were used to prepare rabbit anti-rat erythrocyte membrane serum.

Erythrocytes of normal rats were washed three times in Alsever's pH 7.2 to remove serum components. The packed erythrocytes were then divided into two equal aliquots. Ten volumes of Alsever's solution were added to one aliquot, and ten volumes of a 1/4 percent trypsin solution (see appendix) were added to the other aliquot. Both solutions

were incubated at 37°C for 1/2 hour after which time the cells were lysed by freeze-thawing (-20°C to 56°C) once. The membranes were washed three times to remove the hemoglobin. One volume of membranes (both unaltered and trypsin altered) was suspended in 19 volumes of TBS.

Immune Plasmodium berghei and Normal Rat Serum

Sera from rats recovering from Plasmodium berghei infection were collected by the following technique:

1) The recovering rats were bled by cardiac puncture. No anti-coagulant was used.
2) The blood was quickly transferred to 12-ml centrifuge tubes and allowed to coagulate at room temperature for one hour.
3) An applicator stick was used to break the clot, and the tubes were then placed in the cold (4°C) overnight.
4) The next day the tubes were centrifuged at 1500 x G for 15 minutes to sediment the clot.
5) The sera were then carefully decanted and placed in a water bath set at 56°C for 30 minutes to inactivate the complement.
6) The sera were then placed in small bottles and frozen at -20°C until needed.

Sera from normal rats were collected, inactivated, and stored in the same manner as described above.

Rabbit Anti-Rat Erythrocyte Membrane Serum

To determine the serological purity of the free parasite antigen, anti-rat erythrocyte membrane serum was produced in a 2500 gm white

laboratory rabbit by a series of injections of the rat-erythrocyte membrane antigen. Following a baseline bleeding, 0.15 ml of rat-erythrocyte membrane antigen mixed with 0.15 ml of Freunds Complete Adjuvant (Difco Laboratories, Detroit, Michigan) was injected into the footpads twice a week for four weeks. After the last injection, the animal was rested for one week. Then a sample bleeding was made, and the serum was tested against rat-erythrocyte membrane antigen in a complement-fixation test. All anti-sera taken from the rabbit were collected, inactivated, and stored in the same manner as described in the previous section.

COMPLEMENT-FIXATION TEST PROCEDURE

Introduction

The complement-fixation (CF) test procedure used in this study was the one described by Kent and Fife (1963). The procedures for the standardization of reagents, antigen and anti-sera anti-complementary titer determinations, and the actual complement-fixation test itself were strictly observed. A brief description of each procedure is given below.

Standardization of Reagents

The reagents requiring standardization in the CF test procedure were 1) the buffered salt solution (TBS), 2) the complement, 3) the hemolysin, and 4) the sheep cells. The TBS was routinely prepared from a stock (10X concentration) solution, and the pH was adjusted to 7.3 (see appendix).

The complement was commercially prepared (Grand Island Biological Co., Grand Island, New York) and was furnished in the lyophilized form. Following the addition of the restoring fluid, 1 ml aliquots were pipetted into 12 x 75 mm cuvettes, then quick frozen at -20°C in a CO_2-acetone mixture and stored at -70°C. A sample was then removed, thawed at 4°C and standardized with a Spectrophotometer according to the procedure described by Kent and Fife (1963). On the basis of the titration, dilution of the stock complement was carried out so that a solution containing 5 C' H50/0.3 ml was obtained.

The hemolysin was also commercially obtained (Grand Island Biological Co.) and was furnished as a 1:2 dilution in neutral glycerin. The hemolysin was stored at -20°C. Standardization was accomplished by adding to one unit of complement a standard volume of sheep cells which were sensitized with various concentrations of hemolytic antiserum. Hemolysis was determined using a spectrophotometer, and the results were plotted on graph paper. The optimal concentration of hemolysin was then determined graphically according to the procedure described by Kent and Fife (1963).

The sheep red blood cells were prepared in a 2 percent solution by the following procedure. Sheep erythrocytes at least one week old were washed until the supernatant was clear. Then 2 ml of the packed erythrocytes were added to 98 ml of chilled TBS and mixed thoroughly. Following this, 0.6 ml of the erythrocyte suspension was added to 3.4 ml double-distilled water; the mixture was centrifuged at 1000 x G for 5 minutes; and the amount of hemoglobin released was determined at 550 mμ against a blank of double-distilled water using a Perkin-Elmer Model 139

Spectrophotometer (Perkin-Elmer, Norwalk, Conn.). The following formula was then used to determine the amount of TBS to be added for the final adjustment of the erythrocyte concentration:

$$V_2 = \frac{V_1 \, OD_1}{0.500}$$

where:

V_2 = final volume of erythrocyte-TBS suspension

V_1 = initial volume of erythrocyte-TBS suspension before adjustment

OD_1 = optical density of initial volume of erythrocyte-TBS suspension

A standardized suspension prepared in this manner contained on the average 5×10^8 erythrocytes per ml.

Antigen and Anti-Sera Anti-Complementary Titer Determinations

All antigens and anti-sera used in the CF tests were first tested for their anti-complementary properties. This was accomplished by serially diluting the specimens in TBS and adding 2.5 complement units to each diluted antigen or 5 complement units to each diluted anti-serum. The specimens were then incubated for 18 hours in the cold (4°C). Following incubation, sensitized sheep erythrocytes were added to each tube, the mixtures were incubated at 37°C for 30 minutes; and the degree of hemolysis was read against the controls according to the procedure of Kent and Fife (1963).

The highest dilution of the antigen or antiserum showing complete hemolysis was considered the optimum dilution. All specimens were routinely adjusted to their optimum dilution before use in the actual CF test.

Complement-Fixation Test

Anti-sera (optimum dilution) were serially diluted in TBS and antigen (optimum dilution) was then pipetted into each tube. This was followed by the addition of 5 units of complement to each tube. All tubes were then incubated for 18 hours at 4°C. Sensitized sheep erythrocytes were then added to each tube, and the total reaction volume in each tube was 1.5 ml. The mixtures were incubated for 30 minutes at 37°C, centrifuged at 1000 x G for 5 minutes, and read by visual comparison with standards simulating various degrees of hemolysis. Titers given are the reciprocal of the highest dilution of anti-sera giving less than 65 percent hemolysis.

INFECTIVE TEST OF FREE PARASITES

The infective qualities of the ultrasonically freed parasites were estimated by comparing the pre-patent periods and the mean survival times of mice infected with the free parasites with those given comparable numbers of parasites in infected rat blood. A dilution representing 1×10^6 cells of each specimen was prepared and injected intraperitoneally (IP) into Swiss mice each weighing between 25 and 30 grams. Daily Giemsa smears were then made from each mouse, and the time was recorded in which parasites were first observed in the blood smears. Time for death to occur was also recorded.

The infected blood was drawn from a donor rat, washed three times in Alsever's solution pH 7.2, and a Giemsa smear was made to determine the percent parasitemia. A more critical count was also made in which

the number of parasites per infected red cell was estimated. This was most accurately accomplished by using blood of low parasitemia in which the ring forms predominated and by counting the nuclei of the parasites. The blood was then counted using a hemocytometer, and the appropriate dilution was made to yield approximately 1 x 10^6 parasites per innoculum. The diluted specimen was then kept in the cold (4°C) until 3 hours after the original blood collection. (This is approximately the time required to process the free parasites.) The diluted infected rat blood was then injected IP into a group of ten mice.

The free parasites were prepared and counted by the methods previously described. A dilution was then made in Alsever's solution pH 7.2 to yield approximately 1 x 10^6 free parasites per innoculum. The diluted free parasites were then injected IP into a group of twenty mice.

CHAPTER III

RESULTS AND DISCUSSION

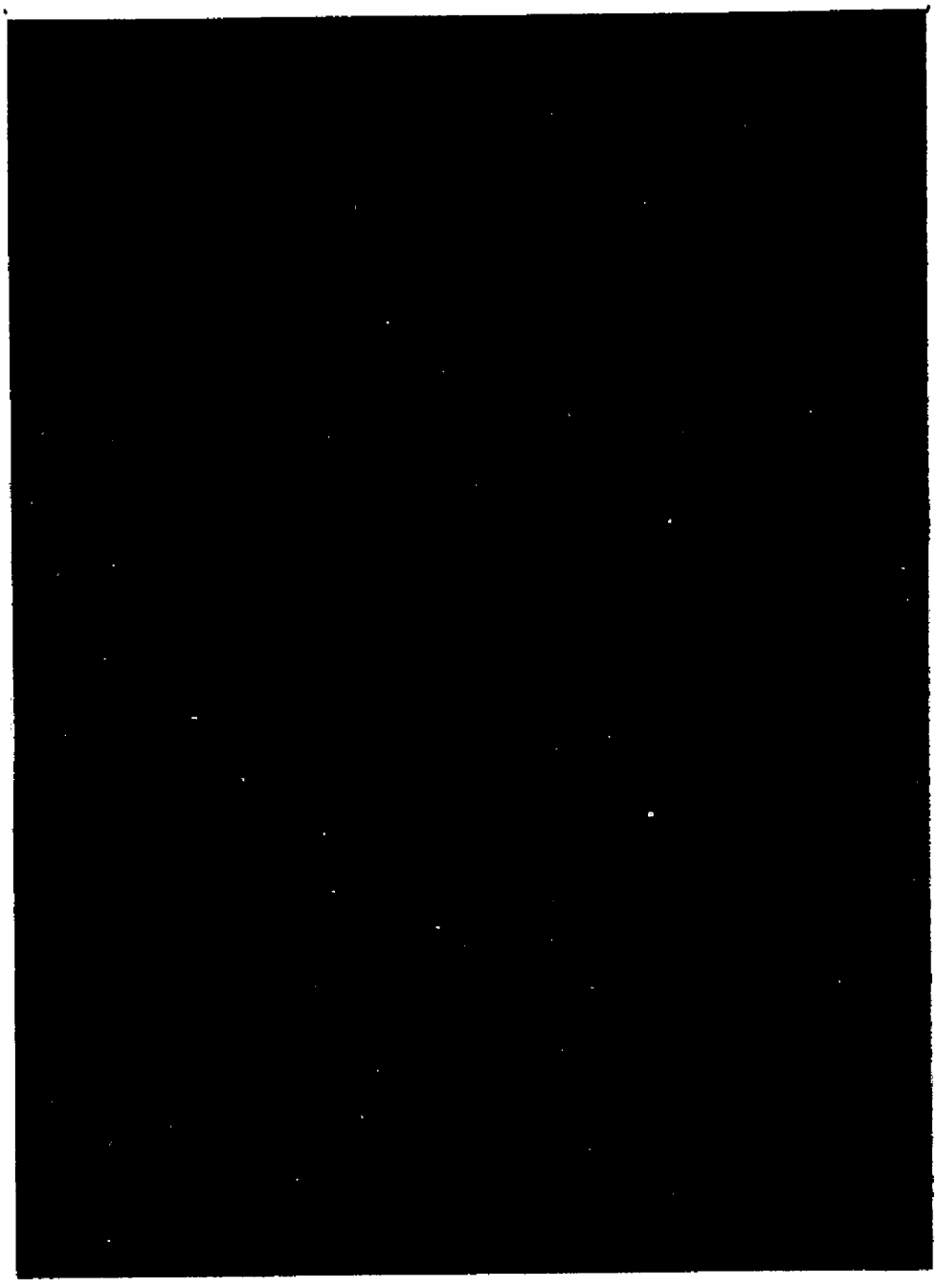
MICROSCOPIC EXAMINATION OF FREE PARASITES

Light and Fluorescence Microscopy

Giemsa-stained smears were made of the free parasites. In these preparations, the free parasites can be seen to retain the staining characteristics of the intraerythrocyte parasites (Fig. 7). The nuclei stain a dark red-purple while the cytoplasm stains a dark blue. Morphologically, the free parasites appear round and range in diameter from about 1 to 3 μm. The smaller forms are most plentiful. Examination of Giemsa-stained preparations did not reveal any evidence that the act of freeing the parasites caused physical damage to them. While the evaluation of Giemsa-stained preparations alone is not sufficient to conclude that the freed parasites were not damaged, the demonstration of normal staining characteristics does strongly suggest that the free parasites are physically unaltered. Lucas and Jamrox (1961) point out that smudged or mechanical damaged cells usually display a change in staining characteristics.

It is most difficult by examination of Giemsa-stained preparations to detect contaminating host erythrocyte membrane material either completely surrounding the parasites or partially attached to them as the membranes are quite thin and stain poorly. Erythrocyte membranes could also collapse around the parasites which then would appear as part of

Fig. 7. Photomicrograph of Giemsa-stained Plasmodium berghei freed from host erythrocytes by ultrasound. Their normal staining characteristics are similar to those of intraerythrocytic plasmodia. Large aggregates of free parasites can be seen. 2,000X.

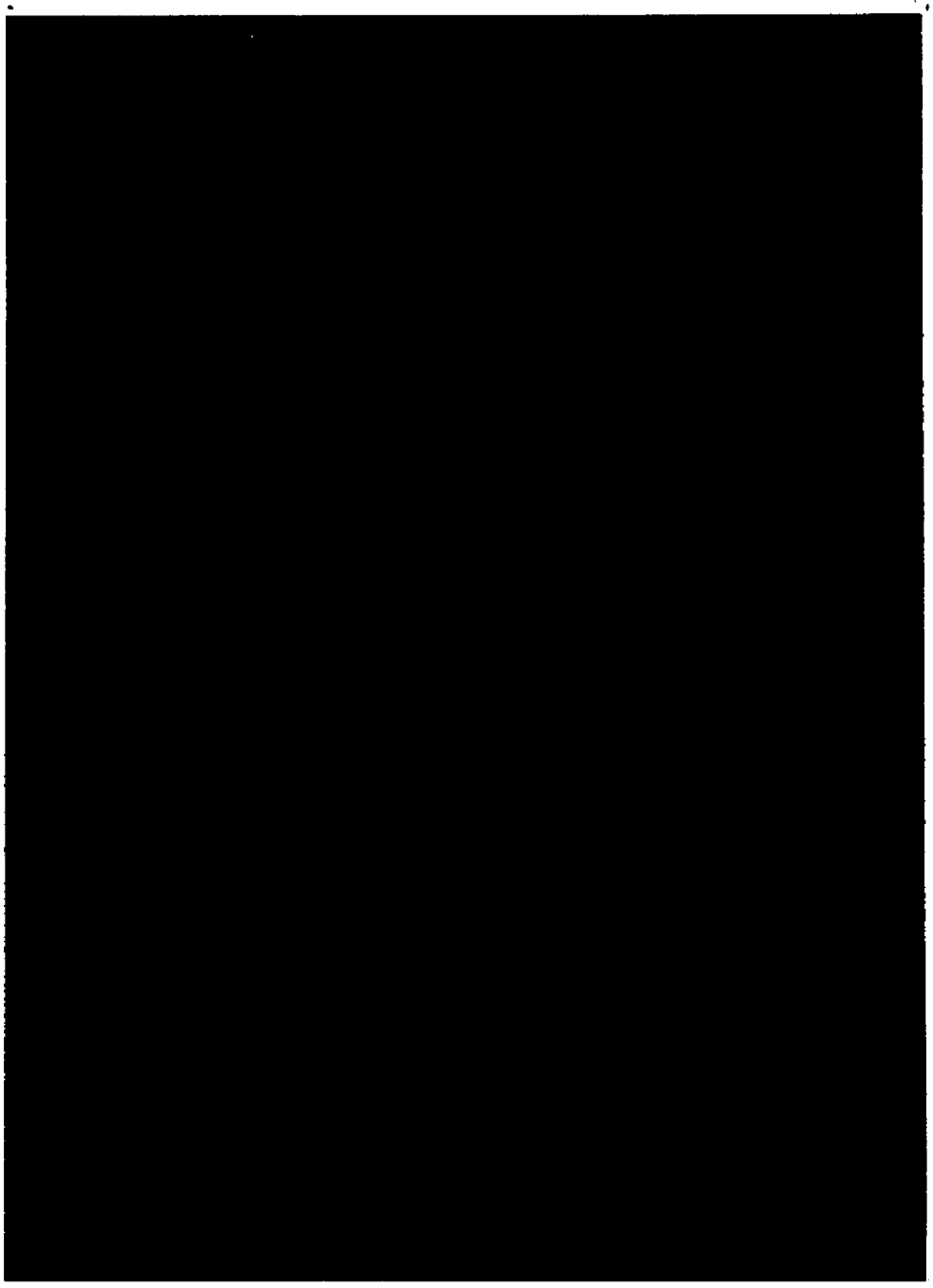


the parasite structure. Cook et al. (1967) have pointed out previously that light microscopic examination is not sufficient to demonstrate the absence of host membrane contamination in a free parasite preparation. The Giemsa-stain technique does, however, provide a quick and reliable method for evaluating the effectiveness of the differential centrifugation procedure for elimination of erythrocytes from the preparations. Erythrocytes stain well, and examination of smears made at each step during the separation procedure showed that virtually all erythrocytes were removed from the free-parasite pellet. The occasional erythrocyte that can be seen in the free-parasite preparations is probably introduced into the preparation when the supernatant is decanted after the initial centrifugation spin.

Examination of acridine-orange-stained preparations by fluorescent microscopy also shows that the free parasites stain normally (Fig. 8). The acridine-orange procedure does not, however, reveal more internal structure than is revealed in the Giemsa-stained preparations. In the acridine-orange-stained preparations, the free parasites appear round as in the Giemsa-stained preparations. The nuclei fluoresce green while the cytoplasm fluoresces a brilliant red. The red fluorescence of the cytoplasm of the free parasites indicates that they have a high content of RNA. The nuclei average about 1/3 μm in diameter and appear to be located peripherally near the outer limiting membrane of the parasite.

Occasional erythrocytes are observed in the acridine-orange-stained preparations as they are in the Giemsa-stained preparations. These

Fig. 8. Photomicrograph of acridine-orange-stained Plasmodium berghei freed from host erythrocytes by ultrasound. The free parasites fluoresce a bright red indicating a high RNA content and the nuclei fluoresce green. 3,000X.

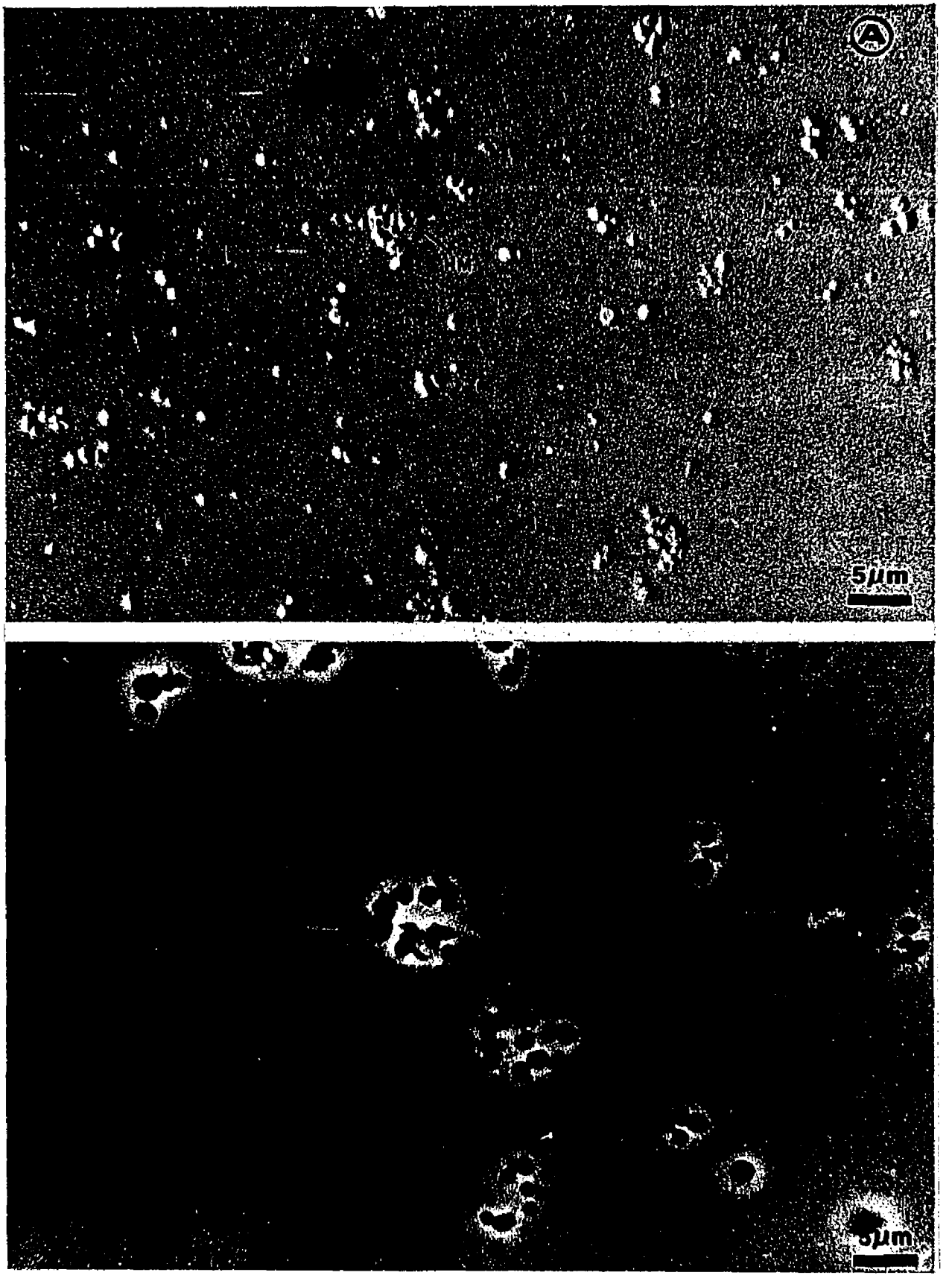


intact erythrocytes are harder to see in the acridine-orange-stained preparations than in the Giemsa-stained ones since they fluoresce a very light green. An occasional damaged free parasite can be observed in the acridine-orange-stained preparations. In these damaged parasites, there is a "streaming" of green fluorescent material from the parasites. This indicates damage to the limiting membrane of the parasite and to the parasite nuclei with concomitant release of nuclear material. Such damaged parasites are observed only rarely, and a slide has to be examined thoroughly to find such damaged parasites. There are less than 1% damaged parasites in the preparation. Damage of the type observed also could occur in preparing the smear. Host cell membranes cannot be observed in the acridine-orange-stained preparations observed with the fluorescence microscope.

The free-parasite preparations were examined by both phase-contrast and interference-contrast microscopy (Fig. 9). Figure 9-A is an interference-contrast photomicrograph, and Figure 9-B is a phase-contrast photomicrograph of free plasmodia. The free parasites appear globular, and no evidence of physical damage to the free parasites can be seen. The free parasites disperse when mixed, but they have a tendency to clump together subsequently. Clumps or aggregates of free parasites can be seen in Figure 9. Figures 7 and 8 also show the clumping of the free parasites. A possible cause of clumping is a low surface charge on the free parasites.

The use of phase-contrast or interference-contrast microscopy proved to be a rapid and reliable method to determine the quality of the

Fig. 9. Interference-contrast and phase-contrast micrographs of Plasmodium berghei freed from host erythrocytes by ultrasound. 9-A. Interference-contrast micrograph of free plasmodia showing clumping of some free parasites and a parasite surrounded by host membrane (HM). 9-B. Phase-contrast micrograph showing free parasites. No parasites surrounded by host membranes are seen in this photomicrograph.

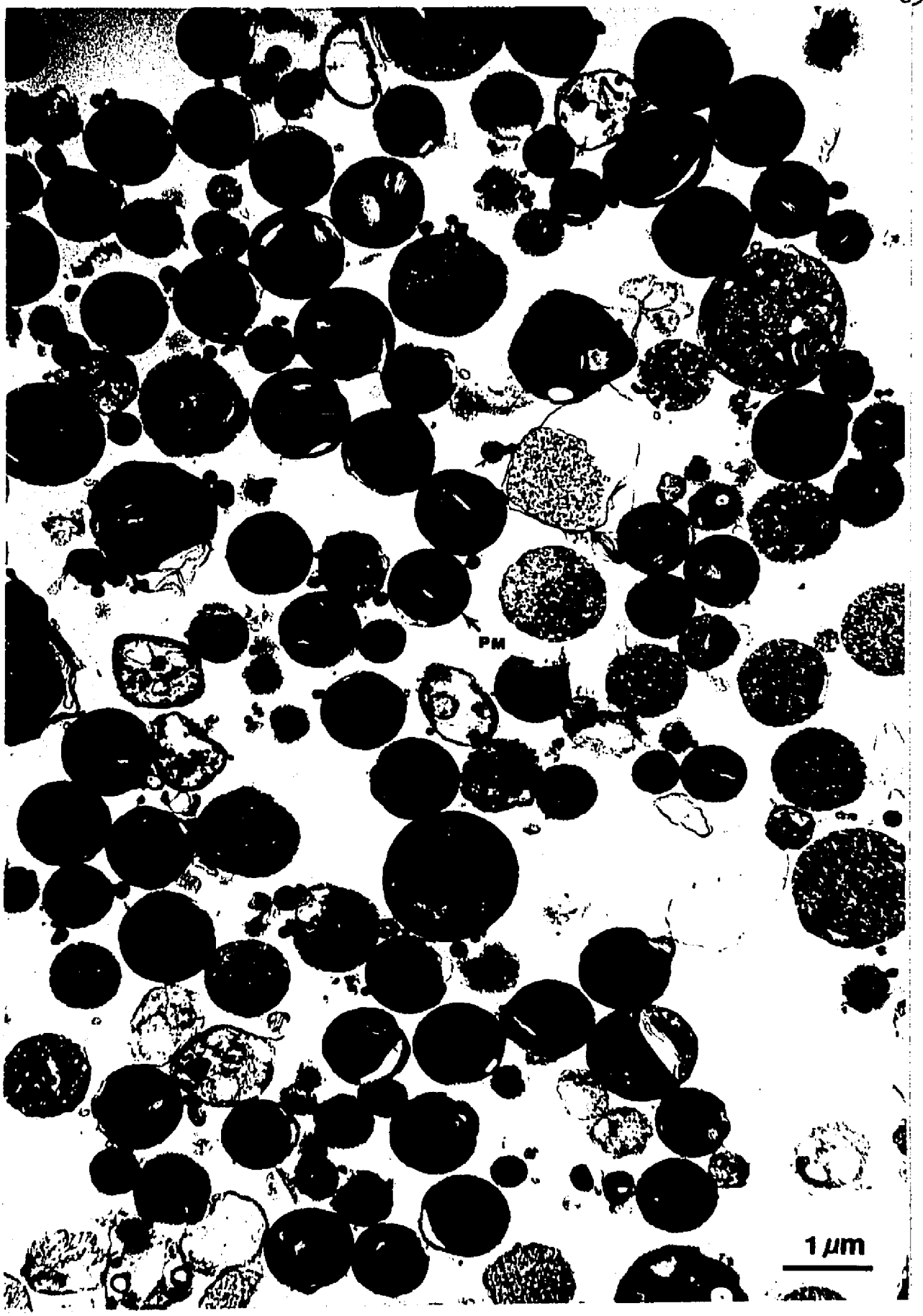

A
5μm
5μm

free-parasite preparations. Ghost cells as well as amorphous granular material could be seen easily by these techniques. For example, the presence of a host membrane (HM) surrounding a parasite is shown in Figure 9-A. Consequently, each batch of free parasites produced was evaluated first by use of phase-contrast or interference-contrast microscopy. In addition, each step in the process of producing free parasites was monitored using one of these microscopic techniques. However, as was pointed out previously, there is a possibility that the entrapping host cell membrane could collapse around the free parasites and thus not be easily detected. Also, microvesicles produced from host membranes by sonication would not be detected by either of these techniques. Only thin-section electron microscopy can adequately determine the presence of these host membrane components.

Electron Microscopy

Figure 10 is a low-power electron micrograph of a representative field of a thin-section of malaria parasites freed from their host erythrocytes by ultrasound. Small merozoites predominate in the field although larger trophozoites (T) are present. The merozoites measure approximately 1 μm in diameter and appear to be bounded by a discrete outer membrane complex (PM). A few damaged parasites (DP) can also be seen. Such damaged parasites appear to have lost the structural integrity of their outer membranes. These parasites could possibly have been damaged by the effects of the cavitating ultrasonic field or possibly by manipulation of the specimen during fixation, dehydration, and embedding.

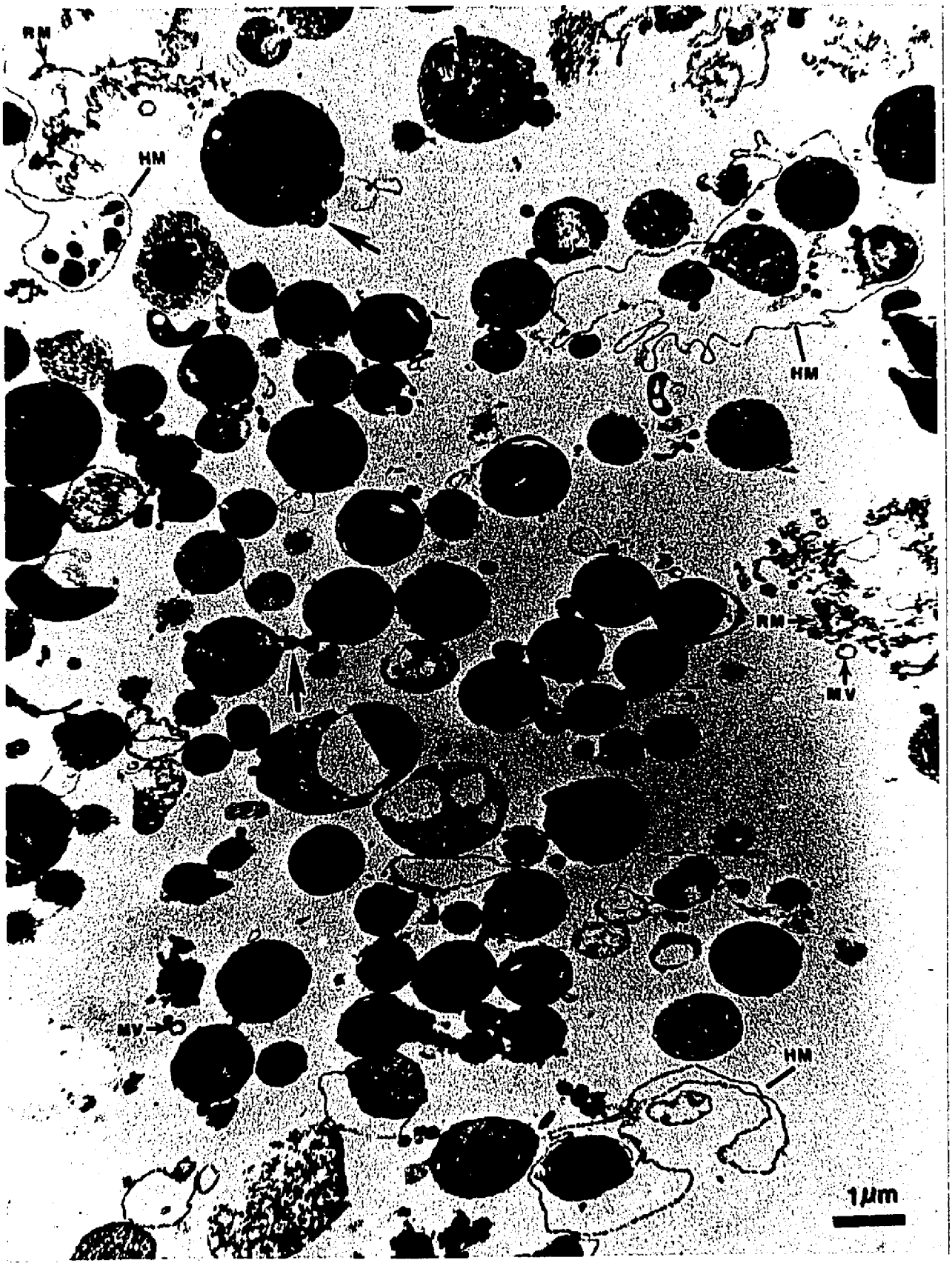
Fig. 10. Low-power electron micrograph of a thin-section of malaria parasites freed from their host erythrocytes by ultrasound. Many merozoites bounded by an outer membrane (PM) are seen as well as some larger trophozoites (T). Some damaged free parasites (DP) are also shown. All parasites are free of entrapping host erythrocyte membranes.


PM
1 μm

All parasites shown in Figure 10 are free of host erythrocyte membranes. Entrapping membranes are not seen in this figure. However, not all of the parasites are freed from their host erythrocytes in these preparations. Figure 11 is a low-power electron micrograph of a thin-section of sonically-freed malaria parasites which shows some parasites completely surrounded by host membranes (HM). The area within the host membranes has the same electron density as the surrounding media indicating complete loss of hemoglobin from the erythrocytes. No internal erythrocyte structure is seen, and the parasites appear to be unattached to the host erythrocyte membrane.

Figure 11 also shows a few microvesicles (MV) and some reaggregated membrane components (RM). Kirk (1968) shows that such microvesicles and reaggregation formations occur in sonicated erythrocyte ghost preparations. This researcher points out that the reaggregation of membrane components is attributed to the presence of certain cations such as Ca^{++}. Rosenberg and McIntosh (1968) also show that sonication of red blood cell membranes breaks these membranes into small vesicles and linear fragments which have intact unit membrane structure. These researchers believe sonic energy serves only to break the membranes into small pieces. Such pieces do not sediment as readily in a high gravitational field as the free parasites, and thus they can be removed easily from a preparation by differential centrifugation. The effectiveness of the differential centrifugation procedure used in this study to separate parasites from host cell contaminates in sonicated preparations can be seen in Figures 10 and 11. In these figures, very few microvesicles and reaggregated membrane components can be seen. Such preparations should

Fig. 11. Low-power electron micrograph of a thin-section of Plasmodium berghei freed from host erythrocytes by ultrasound. Some parasites, however, are not free and are surrounded by host membranes (HM). Small microvesicles (MV) and reaggregated membranes (RM) can be seen as well as bud-like projections protruding from the surface of the free parasites (arrows). A schizont (S) is also shown.

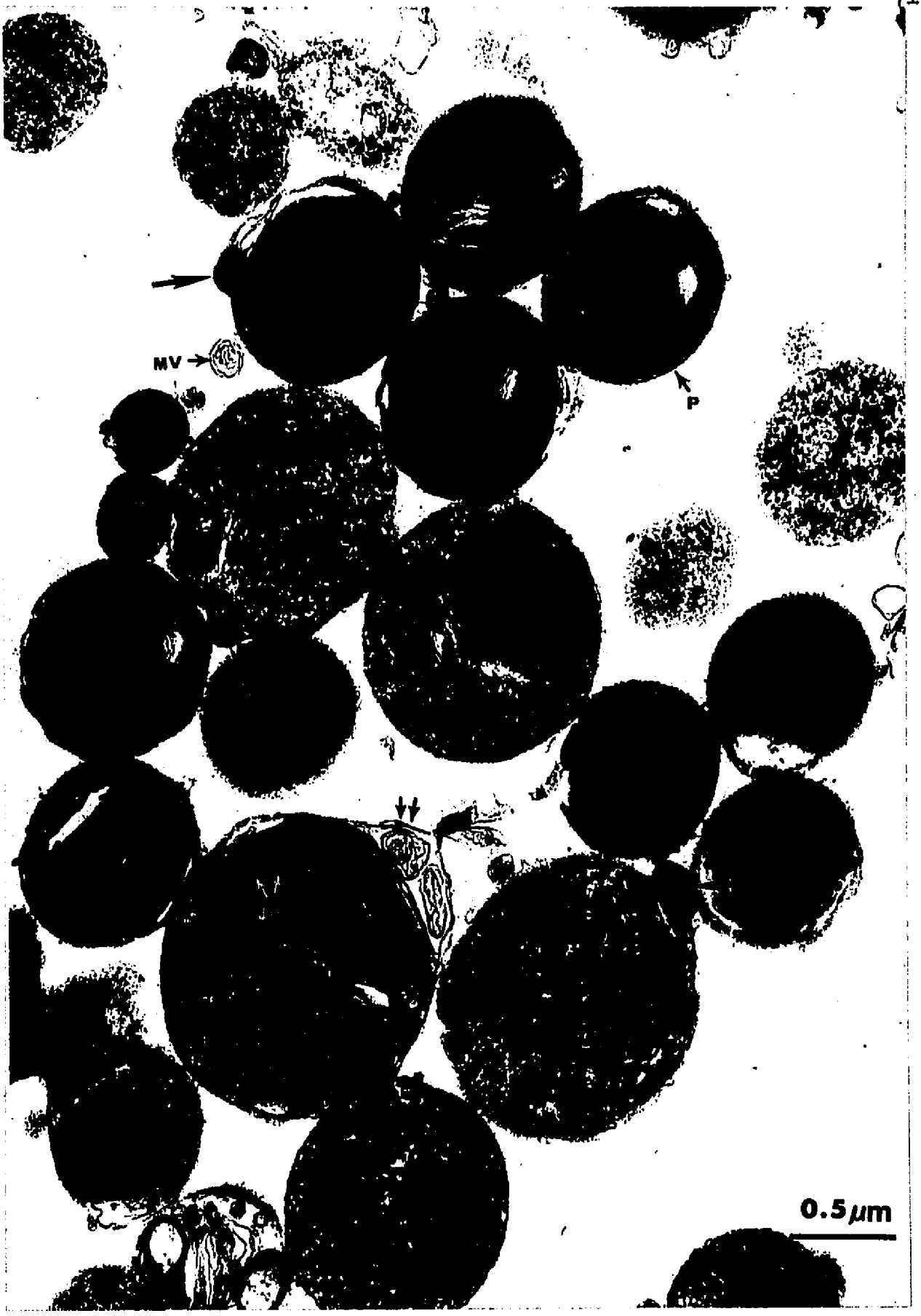

RM
HM
HM
RM
MV
MV
HM
1µm

be satisfactory as antigens for serological tests. However, for studies of the enzymes of the free parasites, further purification should be made to remove these trace amounts of contaminants which are most probably host cell in origin.

Small bud-like projections are seen protruding from the surface of some of the free parasites (Fig. 11--arrows). The large parasite (S) at the top of this electron micrograph appears to have four such structures protruding from its surface and is probably a schizont. The exact number of budding merozoites cannot be determined from thin-section electron micrographs as only a small portion of the parasite's topography is revealed in the section. Other techniques such as carbon replication are needed to determine better the number of these projections. Ladda (1969) in his paper on the fine structure of rodent malaria parasites shows that merozoites develop by budding during schizogony. Aikawa (1971) also shows merozoites in various stages of budding. In the preparation of free parasites examined in this study only the early stages of budding were seen. The absence of the advanced stages suggests that the larger merozoites are "knocked off" by the cavitating ultrasonic field. Such merozoites could possibly be those parasites previously discussed showing damage or structural defects in their limiting membranes.

Figure 12 is a high-power electron micrograph of a thin-section of ultrasonically-freed plasmodia and is included to show the fine structure of the free parasites. The bud-like projections on some parasites are shown (arrows). The outer surfaces of most of the free parasites appear to be smooth. A microvesicle (MV) is shown which is possibly of

Fig. 12. High-power electron micrograph of a thin-section of free malaria parasites showing a microvesicle (MV); bud-like projections (arrows) on the surface of some parasites; the pellicular complex (P) of the merozoites; an outer membrane (double arrows) of a trophozoite probably of host cell origin; a trophozoite (T) without this outer membrane; double membrane bodies (db); nuclei (n); and numerous electron dense structures in the cytoplasm and ribosomes.


MV
P
0.5 μm

host erythrocyte origin but could possibly be from a disintegrated parasite. All parasites shown in Figure 12 are free of entrapping host erythrocyte membranes.

Most of the free parasites shown in Figure 12 possess a pellicular complex (P). Aikawa (1971) defines this pellicular complex as a wall of the parasites which delineates the parasite cytoplasm from its surroundings. Aikawa (1967 and 1971) shows by thin-sectioning techniques that the pellicular complex of the erythrocytic merozoites is composed of three layers: a thin outer membrane, a thick interrupted inner membrane, and a row of microtubules. However, the merozoites in Figure 10 do not show the microtubules. These merozoites appear to be surrounded by three discrete membranes, some of which have discontinuities. The less electron-dense trophozoite (T) is surrounded by only one membrane. Aikawa (1971) states that after a merozoite enters a red cell, as the merozoite develops into an early trophozoite, the pellicule complex breaks down. The trophozoite is then surrounded by only a single plasma membrane in addition to the membrane formed by the host cell. The trophozoite (T) shown in Figure 12 apparently lacks this outer membrane formed by the host cell.

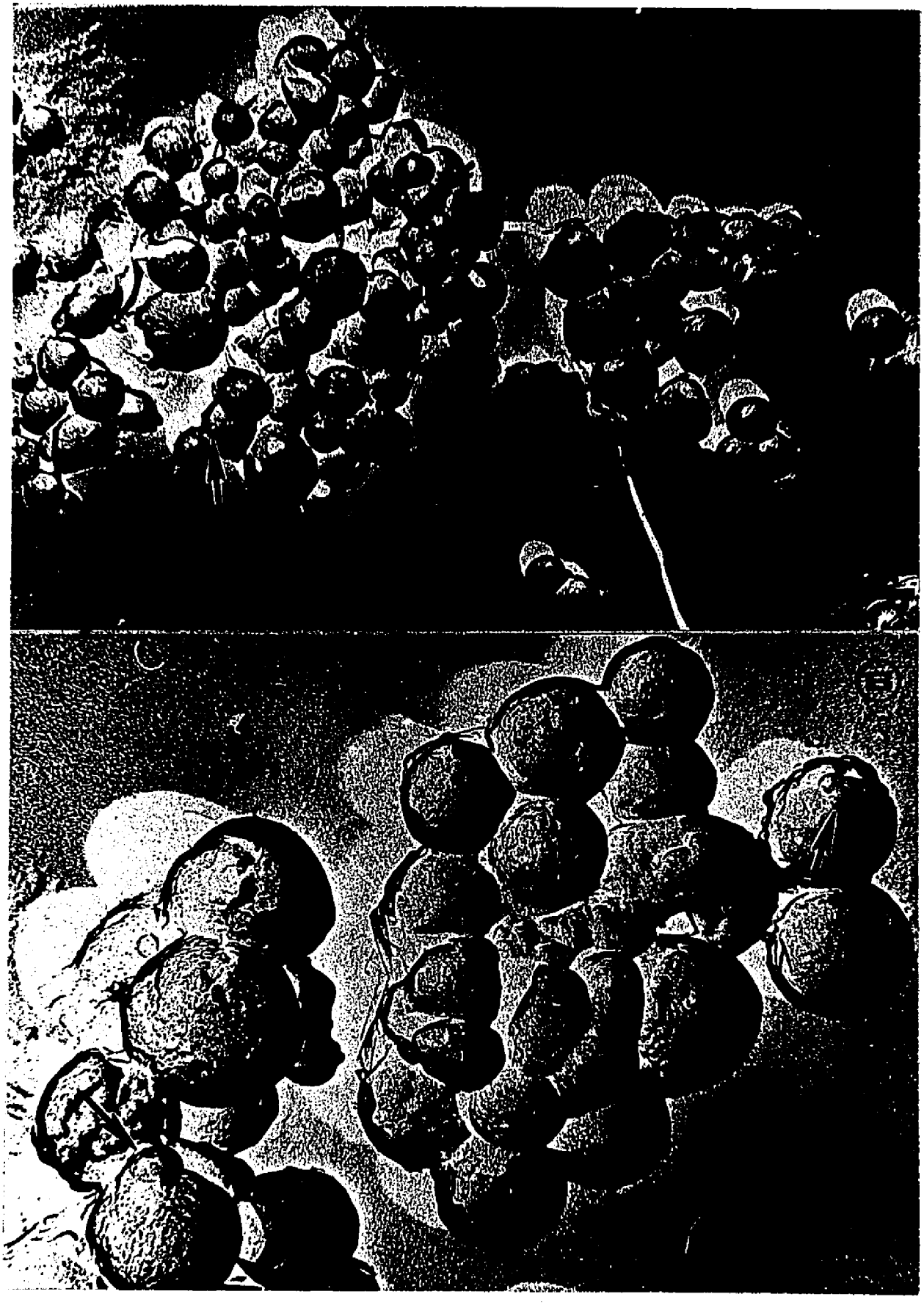
The exact source of the outer membrane is still somewhat controversial although most investigators believe it is of host erythrocyte origin (Ladda, 1969, and Aikawa, 1971). Ladda (1969) proposed that the parasite enters the erythrocyte by a phagocytic type process. Such a method of entry would cause the parasite to be surrounded by a host cell membrane. Bahr (1966) released plasmodia from their host erythrocytes

by proteolytic digestion with pepsin and showed the presence of an outer membrane which appeared to be wrapped tightly around the single outer membrane of the trophozoites. Bahr (1966) feels this outer membrane is of host cell origin. A membrane enveloping a free parasite can be seen in Figure 12 (double arrows). This may be the invaginated erythrocyte membrane which remains around the parasite after entry and would be comparable to the membrane lining the phagocytic vacuole. The use of ferritin-labeled anti-rat erythrocyte membrane antisera would yield information which would answer the question of the origin of this membrane.

The internal structure of the free parasites appears to be well preserved. The presence of loosely aggregated ribosomes throughout the cytoplasm confirms the observation made in the acridine-orange-stained preparations, that the free parasites have a cytoplasm rich in RNA. Various electron dense areas ranging in size from about 0.05 μm to 0.1 μm in diameter, and double-membrane bound bodies (db) approximately 0.1 μm in diameter can be seen in the cytoplasm of some of the free parasites. The nuclei (n) appear to be less electron dense than the cytoplasm and stain evenly. The nuclei are bounded by a double-layered nuclear membrane and are located eccentrically in the parasites. This observation confirmed that made on the acridine-orange-stained preparations that the nuclei are located near the limiting membrane of the parasites.

In order to determine the sizes of the free parasites and to observe their outer surfaces, carbon replicas were made of the preparations. An electron micrograph of a typical carbon replica of parasites freed by ultrasound is shown in Figure 13. Figure 13-A is a low-power electron

Fig. 13. Carbon replica of Plasmodium berghei freed from host erythrocytes by ultrasound. The surfaces of most free parasites resemble an "orange peel." The buds seen on some parasites in thin sections can also be seen in these preparations (arrows). Large clusters of free parasites occur. Some debris is present in both A and B, and what would probably be a damaged parasite (DP) is also shown. 13-A. A low-power electron micrograph. 13-B. A high-power electron micrograph.



micrograph, and Figure 13-B is a higher-power electron micrograph of free parasites. The free parasites range in size from about 1 µm to 3 µm in diameter with the smaller sizes most plentiful.

The free parasites appear in clusters or aggregates in the carbon replica as they do in preparations observed by light microscopy. Some of the free parasites shown in thin sections (Figures 8 and 9) are in direct contact with each other while others are not. However, just because there is no contact in the plane of section of a thin section, one cannot conclude that contact does not occur in some other plane. Figure 13 tends to support the suggestion that most of the free parasites are clumped as only rarely were unclumped parasites observed in the carbon replica preparations.

Most of the free parasites are spherical in carbon replica. This conclusion is compatible with the observations made by light microscopy and the thin-section electron microscopy. Merozoites are generally described as ovoid to elongated, but they round up after entering an erythrocyte (Aikawa, 1971). It is possible that the removal of newly-formed merozoites from their host erythrocytes would cause them to round up even if they had not assumed a spherical shape in the erythrocyte. Virtually every free parasite observed by either light or electron microscopy was spherical.

The outer surfaces of the free parasites can be seen in the carbon replica preparations (Fig. 13). Buds can be seen protruding from the surfaces of some parasites (arrows), and these range in size from about 0.1 to 0.3 µm in diameter. The buds are irregularly placed on the

surfaces of the parasites. The surfaces of the free parasites have an "orange-peel" appearance, and this pebbled surface could possibly result from the adhesion of the outer membrane to the thick interrupted inner membrane of the pellicule complex which Aikawa (1971) describes as a labyrinthine structure. It is obvious from examination of electron micrographs of the carbon replicas as well as the electron micrographs of the thin sections that the membrane system of the parasites is quite complex and changes during the development cycle.

EVALUATION OF FREE PARASITES AS CF TEST ANTIGENS

Protein Assay of Antigens

Protein assays were made by the Biuret technique (Clark, 1964) of the lysed free-parasite antigens, the parasite antigens produced by hypotonic lysis of infected erythrocytes, and the rat-erythrocyte membrane antigens. The results are shown in Table I. The protein assays were made on the 1:1 dilution of the antigens, and the values listed in Table I are averages of several determinations and are expressed as milligrams (mg) of protein per ml. Variations in protein concentrations occurred in different determinations of antigen and were, in part, the result of error in the initial standardization of the antigen to a 5 percent packed volume of parasites.

The protein content of a malarial parasite was estimated by dividing the total protein concentration of a ml of antigen by the number of free parasites in a ml of antigen. The average batch of free-parasite antigen contained 13 mg protein/ml and had average counts of 1×10^{10}

TABLE I

PROTEIN CONCENTRATIONS OF FREE-PARASITE ANTIGEN, LYSED PARASITIZED ERYTHROCYTE ANTIGEN, AND RAT ERYTHROCYTE MEMBRANE ANTIGEN (1:1 DILUTION) AS DEMONSTRATED BY THE BIURET METHOD

Antigen	Average Protein Concentration mg/ml
Free-Parasites	13.0 (3)
Lysed Parasitized Erythrocytes	6.8 (3)
Rat Erythrocyte Membranes	8.5 (2)

( ) = number of determinations

free plasmodia/ml; therefore, each parasite contained about 1.3×10^{-6} µg protein. This estimate agrees quite well with that made by Bahr (1966) who estimated the dry mass of Plasmodium berghei to be between 2.23 and 2.71×10^{-6} µg per parasite for merozoites and trophozoites.

Anticomplementary Titers and Optimum Concentrations of Antigens

The lysed free-parasite, lysed parasitized erythrocyte, and rat erythrocyte membrane antigens were titrated to determine their anticomplementary activity. Typical results are shown in Table II. The anticomplementary titer was defined as that dilution of antigen which demonstrated no fixation of complement in the presence of 2.5 units of complement. Results show the anticomplementary titers of the free-parasite, lysed parasitized erythrocyte, and rat erythrocyte membrane antigens to be 160, 80, and 80 respectively. However, some batches of free parasite antigens had anticomplementary titers as low as 80. The parasite and rat erythrocyte membrane antigens had anticomplementary titers similar to those obtained with the free-parasite antigen, but generally they were one dilution lower. This reduced anticomplementary affect may simply reflect low protein content and the dilute nature of these antigens.

To determine better the optimal concentration of antigen, a block titration was made using the free parasite antigen and antiserum. In this case, both antigen and antiserum were diluted and tested for fixation of complement. The initial dilution of antigen was 1:80 since the particular batch of antigen tested had an anticomplementary titer of 80. The results of the "block-type" titration are shown in Table III. The

TABLE II

ANTICOMPLEMENTARY TITERS OF FREE-PARASITE ANTIGEN, LYSED PARASITIZED ERYTHROCYTE ANTIGEN, AND RAT ERYTHROCYTE MEMBRANE ANTIGEN USING 2.5 UNITS OF COMPLEMENT. RESULTS ARE TYPICAL OF THOSE OBTAINED WITH EACH BATCH OF ANTIGEN.

	Antigen Diluted 1:							
Antigen	5	10	20	40	80	160	320	640
Free-Parasites	4	4	3	2	1	-	-	-
Lysed Parasitized Erythrocytes	4	3	2	1	-	-	-	-
Rat Erythrocyte Membranes	4	3	2	1	-	-	-	-

4 = Complement fixation (no hemolysis)

- = No fixation (100% hemolysis)

TABLE III

BLOCK TITRATION OF FREE-PARASITE ANTIGEN
AND RAT PLASMODIUM BERGHEI ANTISERUM

Antigen Diluted 1:	Antiserum Diluted 1: 2	4	8	16	32	64	128	Antigen Control (2.5 C'H50)
80	4	4	3+	3	2+	2	1+	-
100	4	3+	3	2+	2	1+	1	-
120	3	2+	2	1+	1	1	±	-
140	2+	2	1+	1	1	±	±	-
160	2	1+	1	1	±	-	-	-
Serum Control (5C'H50)	-	-	-	-	-	-	-	

4 = Complete fixation (no hemolysis)

- = No fixation (100% hemolysis)

antigen diluted 1:80 reacted well with the rat anti-Plasmodium berghei serum (titer 64). However, dilution of the antigen resulted in a reduction in serum titer. One two-fold serial dilution of antigen beyond its anticomplementary titer caused a reduction of the titer to two.

The optimal concentration of antigen is considered to be that concentration of antigen giving maximal reaction with a threshold amount of antibody. Therefore, the optimal concentration of the free parasite antigen is a 1:80 dilution of the antigen in the example above. This dilution is also the anticomplementary titer of antigen. Each batch of antigen was titrated to determine its anticomplimentary activity and then diluted to optimal concentration for CF tests.

General CF Test Results

The comparative CF titers of the free-parasite, lysed parasitized erythrocyte, and rat erythrocyte membrane antigens against rat anti-Plasmodium berghei and rabbit anti-rat erythrocyte membrane sera are shown in Table IV. The free parasite antigen reacted strongly with the rat anti-Plasmodium berghei serum (titer 256) while the lysed parasitized erythrocyte antigen reacted poorly with the same anti-serum (titer 4). The rat erythrocyte membrane antigen showed a slight reaction with this antisera, but the reaction was not strong enough to record a titer. This weak reaction may result from blood group antibody in the anti-plasmodia serum which was obtained from a rat infected with infected red cells, or could result from antierythrocyte autoantibodies (Kreier et al., 1966, and Seed and Kreier, 1969). The lysed parasitized erythrocyte and rat erythrocyte membrane antigens reacted strongly with the

TABLE IV

COMPARATIVE CF TITERS OF FREE-PARASITE ANTIGEN, LYSED PARASITIZED ERYTHROCYTE ANTIGEN, AND RAT ERYTHROCYTE MEMBRANE ANTIGEN AGAINST RAT ANTI-PLASMODIUM BERGHEI SERUM AND RABBIT ANTI-RAT ERYTHROCYTE MEMBRANE SERUM

	Antiserum	
Antigen	Anti-Plasmodium berghei	Anti-Rat Erythrocyte Membrane
Free-Parasites	256	64
Lysed Parasitized Erythrocyte	4	256
Rat Erythrocyte Membranes	WR	256

WR = Weakly reactive

rabbit anti-rat erythrocyte membrane serum (titer 256) while the free parasite antigen reacted to a lesser degree with this antiserum (titer 64). The high titer of the lysed parasitized erythrocyte antigen against anti-rat erythrocyte membrane serum shows this preparation to be highly contaminated with host erythrocyte membranes. In fact, the titer is identical with the titer of the rat erythrocyte membrane antigen. The free-parasite antigen reacted with anti-rat erythrocyte membrane serum. Such a reaction would be expected even from a preparation of plasmodia free from their host erythrocytes since the outer membrane of these parasites is probably host cell in origin (Bahr, 1966, and Aikawa, 1971), and host cell materials are in food vacuoles in the parasite (Ristic and Kreier, 1964; Rudzinska et al., 1965; and Aikawa et al., 1966). The presence of some microvesicles and ghost cells in the free-parasite preparations may also contribute to the reaction with anti-erythrocyte antiserum. However, when compared to the lysed parasitized erythrocyte antigen, the free-parasite antigen is superior in that it reacts to a lesser degree with host cell antibodies and to a greater degree with complement-fixing antibodies in rats infected with Plasmodium berghei.

Sera from 15 rats which had been infected with Plasmodium berghei were tested using the free-parasite antigen. These sera were collected from the rats at various times during the recovery period and titrated for complement-fixing antibodies. Sera were also collected from twenty normal rats and tested for complement-fixing antibodies using the free-parasite antigen. The results obtained are shown in Table V. None of

TABLE V

CF TITERS OF NORMAL AND PLASMODIUM BERGHEI-INFECTED RAT SERA USING THE FREE-PARASITE ANTIGEN

Group	No. in Group	Days Post Infection	Titer
Normal rats	20	--	NT
Infected Rats	4	19	128 128 128 256
Infected Rats	3	26	128 256 256
Infected Rats	4	30	64 128 128 128
Infected Rats	4	37	64 64 128 256

NT = No titer at a 1:2 dilution of serum

normal rat sera tested fixed complement while all of the sera from rats infected with Plasmodium berghei fixed complement in the test. Titers vary somewhat among animals depending on individual particularities and the time after infection at which the serum was collected. These titers are generally higher than those reported by others using other antigens.

Correlation between titers reported earlier in the literature and the titer reported here is difficult as in most cases different CF test procedures and different host-parasite pairs were used. For example, Coggeshall and Eaton (1938) reported CF titers as high as 64 in serum from monkeys with Plasmodium knowlesi infection. But these investigators used different quantities of reagents as well as an end point which they defined as the highest dilution which showed fixation of complement. However, some researchers did use the CF procedure of Kent and Fife (1963) or a slightly modified version which was similar to the procedure used in these studies. Bahr (1966) used the method of Kent and Fife and studied CF reactivity of Plasmodia knowlesi antigen extracted with the French Pressure Cell. Although a numerical titer is not mentioned, titers around 80 can be read from their block titration data of antigen and antisera. D'Antonio et al. (1966b) using a modified Kent and Fife CF procedure also studied CF reactivity with Plasmodium knowlesi antigen prepared by a French Pressure Cell technique. These investigators state that the antigen reacted strongly with antisera, and a titer of 32 was obtained.

Cross Reactivity Tests Against Other Plasmodia

Antisera from other species of plasmodia were titrated against the free-parasite antigen in an attempt to detect complement-fixing

antibodies which would cross react with Plasmodium berghei. Antiserum specific for Plasmodium gallinaceum, Plasmodium vivax, and Plasmodium falciparum were tested. The antiserum for Plasmodium gallinaceum was taken from a White Leghorn chicken forty days after infection while the two human malaria antisera were obtained through the courtesy of Carol Rotilie of the Bacteriological Laboratories, The Ohio State University Hospital. All three antisera were inactivated and tested for anti-complementary activity. The Plasmodium gallinaceum antisera had an anti-complementary titer of 4, and the Plasmodium falciparum antiserum showed no fixation at a 1:2 dilution, and the Plasmodium vivax antiserum was anticomplementary to a titer of 32. Normal chicken and human sera tested at the same time were not anticomplementary at a 1:2 dilution.

The results obtained with these three species of plasmodia against Plasmodium berghei are shown in Table VI. No titer was observed with the Plasmodium gallinaceum antiserum nor with the Plasmodium vivax antiserum. However, the anticomplementary nature of both these antisera would mask low degrees of reactivity. Therefore, no conclusions can be made with these specimens. The Plasmodium falciparum antiserum showed a weak reaction with the free-parasite antigen (titer 2). Since this antiserum was not anticomplementary, the low level of reactivity was not masked. Normal human sera showed no fixation of complement with the free-parasite antigen.

The weak reaction of the Plasmodium falciparum antiserum with the free-parasite antigen was investigated further. Certain components in the patient's sera could possibly have reacted with the rat erythrocyte membrane components which have already been shown to be present to some

TABLE VI

CF TITERS OBTAINED WITH PLASMODIUM GALLINACEUM, PLASMODIUM VIVAX, AND PLASMODIUM FALCIPARUM ANTISERA USING PLASMODIUM BERGHEI FREED FROM HOST ERYTHROCYTES BY ULTRASOUND AS TEST ANTIGEN

Antiserum	Titer
Plasmodium gallinaceum (4)	NT
Plasmodium vivax (32)	NT
Plasmodium falciparum (NR)	2
Normal Chicken Serum (NR)	NT
Normal Human Serum (NR)	NT

() = Anticomplementary titers

NR = No reaction (no fixation)

NT = No titer (50% hemolysis)

degree in the free-parasite antigen. Therefore, the Plasmodium falciparum antiserum was titrated against the rat erythrocyte membrane antigen. No fixation of complement was observed, thus reaffirming the weak cross reactivity observed between anti-Plasmodium falciparum and Plasmodium berghei.

To this reviewer's knowledge, no cross reactivity has been reported in the literature between Plasmodium berghei and the human malarias. Although antisera to human malaria have been reported to react with antigens prepared from the monkey malaria Plasmodium knowlesi (D'Antonio, 1966b), more comprehensive tests are needed before definite conclusions can be made about shared antigens in Plasmodium berghei and Plasmodium falciparum.

Thermal Stability of the Free-Parasite Antigen

A preparation of optimumly diluted free-parasite antigen was placed in a water bath heated at 56°C. Samples were subsequently removed each hour for three hours and titrated for CF activity. In addition, samples of the free-parasite antigen were heated to 100°C for 5 and 10 min. periods and tested against a standard antiserum.

The results of this thermal stability study are shown in Table VII. It can be seen that the activity of the free-parasite antigen is greatly reduced by heating at 56°C for 1 hour (titer <2) and that all reactivity was lost within 2 hours when the antigen was heated at 56°C. The activity was also lost when the antigen was heated to 100°C for 5 min. D'Antonio et al. (1966a) reported the Plasmodium knowlesi isolated by a French Pressure Cell did not lose complement fixing activity after

TABLE VII

THERMAL STABILITY OF THE FREE-PARASITE ANTIGEN AT 56°C AND 100°C

Time	Temperature	CF Titer
0	---	64
1 hr.	56°C	<2
2 hrs.	56°C	NT
3 hrs.	56°C	NT
5 min.	100°C	NT
10 min.	100°C	NT

NT = No titer

heating at 56°C for 1 hour. These investigators reported only slight loss of serological activity to be caused by heating the antigen at 100°C for 5 min. However, their antigen was fractionated by passage through a Sephadex G-200 column, and this may have contributed to the stability of their antigen. Stability of Plasmodium knowlesi and Plasmodium berghei may, of course, be inherently different.

Stability of the Free-Parasite Antigen on Storage

To determine the stability of the free-parasite antigen under different storage conditions, a preparation of free parasites was first harvested, lysed, diluted to the optimal concentration, and tested against rat anti-Plasmodium berghei serum. Equal aliquots of the antigen were then stored at -20°C and -70°C. In addition, portions of the antigen were lyophilized using a Thermovar Model FD-2 Freeze Dryer (Thermovar Industries Corp., Copiague, New York). The results obtained after 1 1/2 months of storage are shown in Table VIII. The titer was reduced from 64 to 8 during storage at both -20°C and -70°C while the lyophilized antigen did not react with the antiserum. The lysed free-parasite antigen is not stable when stored at either -20°C or -70°C or when lyophilized.

The effect on stability of addition of 2% polyvinylpyrrolidone (PVP) to the antigen was studied as this chemical has been reported to stabilize malarial antigen (Fife, 1971). The results of tests on lysed free-parasite antigen stored for 1 month at -20°C or lyophilized with and without PVP are shown in Table IX. The addition of 2% PVP did preserve CF activity in lyophilized lysed antigen (titer 8) but appeared to be

TABLE VIII

STABILITY OF THE FREE-PARASITE ANTIGEN STORED AT -20°C, -70°C, AND LYOPHILIZED FOR 1 1/2 MONTHS

Storing Condition	Titer
Before Storage	64
-20°C for 1 1/2 mo.	8
-70°C for 1 1/2 mo.	8
Lyophilized and Stored for 1 1/2 mo.	NT

NT = No titer

TABLE IX

THE EFFECT OF 2% POLYVINYLPYRROLIDONE ON THE STABILITY OF THE FREE-PARASITE ANTIGEN STORED AT -20°C AND LYOPHILIZED FOR 1 MONTH

Storing Condition	Titer
Before Storage	64
-20°C for 1 mo. (No PVP)	16
-20°C for 1 mo. (PVP)	NT
Lyophilized and Stored for 1 mo. (No PVP)	NT
Lyophilized and Stored for 1 mo. (PVP)	8

NT = No titer

detrimental to the stability of lysed antigen stored at -20°C. The addition of 2% PVP to rabbit liver antigen before lyophilization also preserve CF activity but appeared to be detrimental to the antigen if it was stored at -20°C in aqueous solutions. Although the PVP helped preserve the activity of the lyophilized antigen, the antigen was more stable if it was simply frozen without PVP.

Intact free-parasites frozen at -20°C without PVP were more stable than lysed free parasites stored under the same conditions. The intact frozen parasites stored for 3 weeks showed a reduction of CF activity of less than one dilution. The free-parasite antigens used in most titrations in this study were harvested, quick-frozen, and stored no longer than three weeks at -20°C. For use, the antigens were removed from the -20°C storage chest, lysed as previously described in Chapter II, and diluted to the optimal concentration. This procedure gave satisfactory results as evidenced by the high titers obtained. However, for the production of antigens which may be used commercially for diagnostic CF tests, a more stable antigen would be desirable. Tests must be made of additional stabilizing agents including anti-oxidants such as asorbic acid and glutathione and of the effect on stability of fractionation of antigens.

Other Studies of the Free-Parasite Antigen

Since the free-parasite antigen appeared to be sensitive to freezing, the effect on the antigenicity of the cycles of freezing and thawing used to lyse the parasites was studied. A preparation of intact free plasmodia stored at -20°C was removed from the freezer and thawed at

room temperature. The preparation was then examined, and many intact free parasites were present. The supernatant solution appeared a light brown color after centrifugation indicating lysis of some parasites. This preparation (pellet plus supernatant) was then diluted to optimal concentration and tested for CF activity without subsequent lysis of the free parasites. Results showed the antigen preparation reacted well with homologous antisera. However, in comparison to the free-parasite antigen lysed by the method described in Chapter II, the CF activity was slightly reduced (1 dilution). Therefore, it appears that the three cycles of rapid freezing and thawing increase the CF reactivity of the free-parasite antigen.

Preparations of intact free plasmodia were removed from storage at -20°C and thawed at room temperature. The preparations were centrifuged (500 x G for 10 min.), and the light-colored Alsever's solution containing supernatant was removed. Then TBS pH 7.3 was added to the packed parasites, and the parasites were lysed by the procedure outlined in Chapter II. The antigens were then diluted and tested. The anticomplementary titers of these preparations were lower than those of the "complete" free-parasite antigen and averaged about 64. The protein content also was lower and ranged from 5.2 to 7.9 mg protein/ml. Antigen preparations prepared by discarding the fluid in which the parasites were frozen had titers against homologous antiserum which were on the average one dilution lower than antigens which included this fluid. The CF activity is maximal with antigens prepared by lysing the whole crude free-parasite suspension. Mahoney et al. (1966) found CF activity mostly in the insoluble fractions of Plasmodium knowlesi antigen. Our

initial results indicate that considerable activity resides both in soluble and in particulate materials.

INFECTIVITY OF FREE PARASITES

The infectivity of the free parasites was determined by injecting two groups of mice, one with Plasmodium berghei-infected rat erythrocytes and the other with approximately equal numbers of sonically-freed parasites. The prepatent periods and survival times were then recorded for mice of each group (Table X). The mean prepatent period of the mice inoculated with Plasmodium berghei-infected rat erythrocytes was 3.0 days, and the mean death time was 8.2 days. The mean prepatent period of the mice receiving the sonically-freed Plasmodium berghei was 3.9 days, and the mean death time was 10.3 days. All mice inoculated in this study died.

The sonically-freed parasites are infectious. The prepatent periods and survival times for mice given free parasites were somewhat longer. This result would indicate that there was some loss of infectivity as a result of the sonic treatment. The longer prepatent period and time to death observed in the mice infected with free parasites may, in part, be a result of damage to parasites by ultrasound. However, Moulder (1962) suggests that intracellular parasites may have permeable membranes and lose vital constituents when removed from cells even if the membranes are not damaged. Some parasites were probably phagocytized and destroyed after injection in vivo also. Trubowitz and Masek (1968) have reported that parasites free of erythrocytes are at greater risk of

TABLE X

COMPARATIVE INFECTIVITY IN MICE OF PLASMODIUM BERGHEI-INFECTED RAT ERYTHROCYTES AND PLASMODIUM BERGHEI FREED FROM HOST ERYTHROCYTES BY ULTRASOUND

Group	Dose (No. Parasites)	Mean Day Of Prepatent Period	Mean Day Of Death	No. Survivors/ No. Infected
Mice infected with *Plasmodium berghei*-infected rat erythrocytes	1×10^6	3.0	8.2	0/10
Mice infected with *Plasmodium berghei* freed by ultrasound	1×10^6	3.9	10.3	0/20

ingestion than those parasites contained in erythrocytes. Martin et al. (1971) reported an increased survival time in mice injected with parasites which were freed from erythrocytes by ammonium chloride lysis of Plasmodium berghei-infected erythrocytes. These investigators suggest that phagocytosis and destruction of some of the free parasites probably accounted for the increased survival time. The survival time reported by Martin et al. (1971) was 11 days for an inocula of 5×10^6 free parasites. It appears that the sonically-freed parasites are less damaged than those freed by ammonium chloride lysis.

The free parasites obtained by the continuous-flow system of sonication are infectious. Thus not only are the parasites morphologically undamaged grossly, but they are sufficiently undamaged in their fine structure to be functional. These free parasites may be used in studies involving drug therapy, physiology, and attempts to cultivate malaria parasites on artificial media.

CHAPTER IV

SUMMARY OF RESULTS

MORPHOLOGICAL EXAMINATION

The continuous-flow sonication system developed in this study was suitable for production of large quantities of intact plasmodia free from their host cell membranes. The free parasites were harvested by simple differential centrifugation. The efficiency of the technique for producing free parasites was determined by examination of the free-parasite preparations using various microscopic techniques. Each microscopic technique employed revealed that free plasmodia were released. Only electron microscopy proved unequivocally the effectiveness of separation of parasites from host erythrocytes (Figs. 10 and 11). Most of the parasites shown in Figures 10 and 11 are free of host membranes and are physically unaltered. High-power electron micrographs revealed that the internal structure of the parasites is well preserved (Fig. 12).

The various light microscopic techniques revealed certain characteristics of the free plasmodia not revealed by the electron micrographs. The Giemsa-stained free parasites showed normal staining characteristics as did the free parasites which were stained with acridine-orange. The staining properties of the free parasites suggest that most of these parasites are unaltered morphologically and chemically. The acridine-orange-stained preparations showed that some free parasites were damaged as nuclear material was observed streaming from them.

A few contaminating erythrocytes were observed in the Giemsa-stained smears as well as in the acridine-orange-stained smears of the preparations. Similarly, a few erythrocytes were observed in the parasite preparations with the phase- and interference-contrast microscopes. Erythrocyte membranes could not be detected in the Giemsa-stained preparations because they do not stain by this technique. However, they could be detected by use of the phase- or interference-contrast microscope. The phase-contrast microscope proved to be an efficient tool for monitoring the procedure for harvesting the free plasmodia as well as providing a means of estimating the purity of the free parasite preparation.

The free parasites were spherical and averaged about 1 μm in diameter. Smaller and larger forms were also observed. The free parasites aggregated and formed clusters (Figs. 7, 9, and 13). It is possible that they have low surface charges. The membrane complex of the free merozoites was different from that of the free trophozoites. The merozoites possessed a pellicular complex which was absent from the trophozoites (Fig. 12). In general, the membranes were undamaged by the cavitating ultrasonic field; however, some free parasites did show membrane damage. The continuous-flow system which was adjusted so that the parasites were removed from the field as they were released from the erythrocytes was probably the factor that prevented damage to the parasites.

The surfaces of the free parasites resembled an "orange peel." The pebbled surface was probably due to the characteristics of the thick interrupted inner membrane of the pellicular complex. Small buds,

possibly merozoites, could be seen (Figs. 10, 11, 12, and 13) protruding from the surface of some free parasites. Larger buds were not observed; and these forms, if they occurred, were probably "knocked off" in the cavitating ultrasonic field. Such dislocated parasites may be the damaged parasites observed in the electron micrographs (Fig. 10).

Immunological Examination

The free parasites were evaluated immunologically by using a standardized CF test. The lysed free-parasite antigen, a lysed parasitized erythrocyte antigen, and a rat-erythrocyte membrane antigen were studied comparatively. The lysed parasitized erythrocyte antigen was produced by the procedure of Dulaney (1944) and served as a control. The rat erythrocyte membrane antigen was used as a control and also for immunizing rabbits to obtain anti-rat erythrocyte membrane serum. This serum was then used to determine the presence of host erythrocyte membrane components in the free-parasite antigen.

The free-parasite antigen (1:1 dilution) had approximately 13.0 mg protein/ml. The free-parasite antigen was anticomplementary at low dilutions, but not when properly diluted. Block type titration (Table III) showed the optimal antigen dilution was that dilution which eliminated anticomplementary activity.

The lysed parasitized erythrocyte antigen reacted poorly with antiserum to plasmodia but reacted strongly with anti-rat erythrocyte membrane serum. In fact, this antigen reacted to the same degree with anti-rat erythrocyte membrane serum as did the rat erythrocyte membrane antigen. This shows that the parasite antigen is highly contaminated

with host erythrocyte membranes. The slight reaction observed with the free-parasite antigen also shows the presence of some host cell contaminates.

The serum of none of the normal rats tested fixed complement with the free-parasite antigen while all sera from rats recovering from acute Plasmodium berghei infection fixed complement with the antigen (Table V). Titers varied among the animals tested and with the stage of recovery of the donor rat. The Plasmodium berghei antigen reacted weakly with Plasmodium falciparum antiserum but not with Plasmodium gallinaceum or Plasmodium vivax serum in one set of preliminary tests.

Lysates of free Plasmodium berghei were unstable when stored frozen at -20°C and -70°C. The CF reactivity was lost when the antigen was heated at 56°C for periods over 1 hour (Table VII). The reactivity was greatly reduced (titer<2) when the antigen was heated at 56°C for 1 hour. Boiling for 5 min. completely destroyed the CF reactivity of the lysed antigen. The antigen reactivity was best preserved by freezing (-20°C) the intact free parasites. The reactivity was reduced when the lysed antigen was stored at -20°C and -70°C. Lyophilization of the antigen in the absence of protective agents destroyed the CF activity, but the addition of 2% PVP to the antigen before lyophilization partially protected it (Table IX).

That most of the parasites freed by the continuous-flow system of sonication were infectious was shown by comparing the prepatent periods and survival times in groups of mice injected with free parasites or Plasmodium berghei-infected rat erythrocytes containing a comparable

number of parasites. The prepatent periods and survival times of mice given free parasites were only slightly longer than those of mice given parasitized erythrocytes (Table X).

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Conclusions:

1. Plasmodium berghei may be freed from their host erythrocytes with minimum damage and debris formation by use of a continuous-flow system of sonication. The continuous-flow system assures that each erythrocyte receives approximately equal exposure to ultrasonic energy and that the free parasites are removed from the cavitating ultrasonic field before they are damaged.
2. The development of a flow chamber in which there was direct contact of the specimen with the transducer probe tip made possible the use of low sonic power levels and rapid flow rates. Consequently, large quantities of infected blood were processed quickly and efficiently.
3. It was determined that power level settings which achieved approximately 80 percent red cell breakage produced the maximum number of free parasites. Higher power level settings producing greater red cell breakage caused increased damage to the free parasites at the flow rate tested.
4. The ultrasonically-freed parasites were harvested easily by differential centrifugation. The erythrocytes not lysed by the cavitating ultrasonic field were easily removed by low speed centrifugation while the free parasites were sedimented

by higher gravitational forces. The ghost cells and erythrocyte membrane fractions sedimented more slowly than the free parasites and were removed from the free parasites by washing and spinning the free parasites at 600 x G for 15 minutes.

5. The free parasites were spherical in shape and stained as intracellular parasites stain. Average size of the free parasites was 1 µm in diameter.
6. Thin-section electron microscopy demonstrated unequivocally that most of the parasites were freed from the host erythrocyte membranes; and that in the process, they were not damaged by the cavitating ultrasonic field. The membranes and internal structure were normal in most free parasites.
7. Diluted suspensions of lysed free parasite preparations reacted well with homologous antisera in complement-fixation tests. No normal sera tested fixed complement to the antigen. The free parasite antigen was anticomplementary, but at low dilutions was highly antigenic when diluted beyond the anticomplementary titer.
8. The free parasite antigen contained some host erythrocyte components as demonstrated by reactivity with anti-rat erythrocyte membrane sera. These host membrane components did not affect the reactivity of the free parasite antigen with homologous antisera.
9. The free-parasite antigen was destroyed by heating to 56°C. Lysed free-parasite antigen was not stable when frozen or

lyophilized. The addition of 2% PVP did not stabilize the frozen free parasite antigen but did stabilize the lyophilized preparations. The antigen was most stable when intact free parasites were stored frozen.

10. Most of the ultrasonically-freed Plasmodium berghei were infectious.

Recommendations:

1. The free parasites should be examined by freeze-etch techniques. This technique would provide information about the membrane systems of the parasites.
2. The free parasites should be examined using ferritin labeled antibody techniques. The use of ferritin labeled anti-rat erythrocyte membrane serum would provide evidence as the origin of the parasites' outer membrane.
3. A series of sera from animals infected with other diseases should be tested to ascertain the types and percentages of false positives obtained with the free-parasite antigen. Large numbers of sera from animals infected with other species of plasmodia should be tested to determine possible cross reactivities with Plasmodium berghei. The antibody response curves of animals infected with plasmodia should be determined.
4. Other serological tests such as precipitation-in-gel tests, indirect fluorescent antibody tests, hemagglutination tests, and slide flocculation tests should be examined utilizing the free parasite preparation.

5. Fractionation and purification should be undertaken to eliminate residual erythrocyte material and to attempt to obtain stable antigenic fractions. Such techniques might include the use of Sephadex G-200 and DEAE cellulose gels. Antigenic analysis should then be undertaken with the fractionated antigens.
6. Immunization with purified free-parasite extracts should be investigated.
7. The continuous-flow sonication system should be tested with other malaria parasites such as Plasmodium knowlesi. In addition, attempts to isolate other hemotrophic parasites such as Babesia should also be made using this system. These other antigens should then be tested in homologous and heterologous systems for complement fixation.

APPENDIX

A. Acridine Orange Buffer Solution pH 9

1. Monobasic potassium phosphate (KH_2PO_4) . . . . . 9.078 gm

   Oxalic acid . . . . . 8.404 gm

   Distilled water qs . . . . . 1,000 ml

2. Borax . . . . . 25.480 gm

   Distilled water qs . . . . . 1,000 ml

Mix 1 part No. 1 with 3 parts No. 2 to obtain buffer solution pH 9. Buffer should be freshly prepared before use, but No. 1 and No. 2 are stable indefinitely.

B. Acridine Orange Stock Solution (1:1,000 dilution)

Acridine orange (Will Scientific, Inc., Columbus, Ohio) 0.1 gm

Distilled water qs . . . . . 100 ml

Add 38 ml of acridine orange buffer solution pH 9 to 2 ml of acridine orange stock solution diluted 1:1,000 to obtain acridine orange fluorochrome diluted 1:20,000. Prepare 1:20,000 solution before each use.

C. Alsever's Solution pH 7.2

Citric acid ($H_3C_6H_5O_7 \cdot H_2O$) . . . . . 0.6 gm

Trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) . . . . . 8.0 gm

Sodium chloride (NaCl) . . . . . 4.2 gm

Anhydrous dextrose ($C_6H_{12}O_6$) . . . . . 20.6 gm

Double distilled water qs . . . . . 1,000 ml

Adjust pH to 7.2 with 1N sodium hydroxide solution.

D. Dehydrating Alcohols (35, 50, 70, and 95 percents)

1. 35 percent ethyl alcohol +0.1% $CaCl_2$

Ethyl alcohol absolute (CH_3CH_2OH)	35 ml
Distilled water qs	100 ml

Add 10 drops of 0.1 M calcium chloride ($CaCl_2$) solution.

2. 50 percent ethyl alcohol +0.1% $CaCl_2$

Ethyl alcohol absolute (CH_3CH_2OH)	50 ml
Distilled water qs	100 ml

Add 10 drops of 0.1 M calcium chloride ($CaCl_2$) solution.

3. 70 percent ethyl alcohol

Ethyl alcohol absolute (CH_3CH_2OH)	70 ml
Distilled water qs	100 ml

4. 95 percent ethyl alcohol

Ethyl alcohol absolute (CH_3CH_2OH)	95 ml
Distilled water qs	100 ml

E. EDTA Anticoagulent

Disodium ethylenediamine tetraacetate dihydrate	
$((NaOOCCH_2)_2NCH_2CH_2N(CH_2COOH)_2 \cdot 2H_2O)$	5.0 gm
Phosphate buffered saline pH 7.3	100.0 ml

Concentration of EDTA is 1 mg/ml whole blood.

F. Epon 812 Embedding Mixture

Epon 812 epoxy resin (Ladd Research Industries,	
Burlington, Vermont)	57.55 gm
Dodecenyl succinic anhydride tech. (Fisher Scientific	
Co., Fair Lawn, N. J.)	38.50 gm

F. Epon 812 Embedding Mixture (Cont'd)

Nadic methyl anhydride tech. (Fisher Scientific Co.) .	29.30 gm
Dimethyl amine methyl phenol (Ladd Research Industries)	1.90 gm

Mix well, keep covered to avoid moisture contamination.

G. Glutaraldehyde Solution (1.25%)

Glutaraldehyde solution 50% w/w biological grade (Fisher Scientific Co.)	2.5 gm
1/15 M phosphate buffer pH 7.3 with 4% sucrose	97.5 gm

Make up fresh before use to avoid bacterial contamination.

H. Kolmer's Counting Fluid

Formalin (40%)	2 ml
Glacial acetic acid	2 ml
Double distilled water	96 ml

Mix well.

Carbol fuschin	2 ml

Mix and filter.

I. Osmium Tetroxide Fixative (1%)

Osmium tetroxide (OsO_4) (Mallinckrodt Chemical Works St. Louis, Mo.)	0.5 gm
1/15 M phosphate buffer pH 7.3 containing 0.05 mg of $CaCl_2$ per ml	50.0 ml

Wash outside of glass ampule with distilled water, break glass ampule in flask containing the phosphate buffer, stir for 1 1/2-3

I. Osmium Tetroxide Fixative (1%) (Cont'd)

hrs. Keep flask tightly stopped with rubber stopper lined with aluminum foil. Prepare and mix fixative under hood. Make fresh solution each time when needed.

J. Phosphate Buffer 1/15 M, pH 7.3

1. Monobasic potassium phosphate (KH_2PO_4) . . . . . 9.06 gm
   Double distilled water qs . . . . . 1,000 ml
2. Dibasic anhydrous sodium phosphate (Na_2HPO_4) . . . . . 9.46 gm
   Double distilled water qs . . . . . 1,000 ml

Mix 240 ml of No. 1 with 760 ml of No. 2. If necessary, adjust final pH to 7.3 with 1N sodium hydroxide solution.

K. Phosphate Buffered Saline pH 7.3

Phosphate buffer 1/15 M, pH 7.3 . . . . . 1,000 ml
Sodium chloride (NaCl) . . . . . 8.5 gm

L. Phosphate Buffer 1/15 M pH 7.3 with 4% Sucrose

Phosphate buffer 1/15 M, pH 7.3 . . . . . 100.0 ml
Sucrose ($C_{12}H_{22}O_{12}$) . . . . . 4.0 gm

M. Polyvinylpyrrolidone Solution (2%)

Polyvinylpyrrolidone K-30 (Oxford Laboratories, San Mateo, Cal.) . . . . . 2.0 gm
Triethanolamine-buffered salt solution (TBS) pH 7.3 . 100.0 ml

N. Triethanolamine-Buffered Salt Solution (TBS) Stock 10X Concentration

Sodium chloride (NaCl) . . . . . 75.0 gm

N. Triethanolamine-Buffered Salt Solution (TBS) Stock 10X Concentration (Cont'd)

Hydrochloric acid 1N (HCl)	177.0 ml
Triethanolamine (2,2',2"-nitrilotriethanol) TEA	28.0 ml
$MgCl_2 \cdot 6H_2O$ (4.16 M)	1.2 ml
$CaCl_2 \cdot 2H_2O$ (1.25 M)	1.2 ml

Dissolve the NaCl in 700 ml of double distilled water in a 1-liter volumetric flask. Add the indicated volumes in the order given. Use a 30 ml syringe without needle to measure the TEA. Adjust the volume to 1 liter with double distilled water. Divalent cation concentrations in the stock (10X concentration) should be: 5×10^{-3} M $MgCl_2$ and 1.5×10^{-3} M $CaCl_2$.

O. Triethanolamine-Buffered Salt Solution (TBS) 1X Concentration, pH 7.3

TBS (10X concentration)	100.0 ml
Double distilled water qs	1000.0 ml

Final pH should be 7.3 to 7.4 at 20°C.

P. Trypsin Solution (0.25%)

Trypsin 1:250 (Difco Laboratories, Detroit, Mich.)	0.25 gm
Alsever's solution pH 7.2	100.00 ml

BIBLIOGRAPHY

Ackerman, E. 1952a. Resonant effects of mammalian erythrocytes in ultrasonic fields. Journal of the Acoustical Society of America 24, 118. (an abstract)

Ackerman, E. 1952b. Cellular fragilities and resonances observed by means of sonic vibration. Journal of Cellular and Comparative Physiology 39, 167-190.

Ackerman, E. 1953. Pressure thresholds of biologically active cavitation. Journal of the Acoustical Society of America 26, 1371-1373.

Ackerman, E., and Lombard, D. B. 1955. Ultrasonic destruction of erythrocytes. Institute of Radio Engineers Convention Record 3, 100-108.

Ackerman, E., and Proctor, T. F. 1956. Surface resonances of bubbles and biological cells. Institute of Radio Engineers Convention Record 4, 45-50.

Aikawa, M., Huff, C. G., and Sprinz, H. 1966. Comparative feeding mechanisms of avian and primate malarial parasites. Military Medicine 131, 969-983.

Aikawa, M. 1967. Ultrastructure of the pellicular complex of Plasmodium fallax. The Journal of Cell Biology 35, 103-113.

Aikawa, M. 1971. Parasitological review. Plasmodium: the fine structure of malarial parasites. Experimental Parasitology 30, 284-320.

Angerer, O. A., Barth, G., and Guttner, W. 1951. Uber den werkungsmechanismus biologischer ultraschallreaktionen. Strahlencherapie 84, 601-610.

Bahr, G. F. 1966. Quantitative cytochemical study of erythrocytic stages of Plasmodium lophurae and Plasmodium berghei. Military Medicine 131, 1064-1070.

Binstock, L. 1960. Photographic studies of erythrocytes in ultrasonic fields. Journal of the Acoustical Society of America 31, 1583 (an abstract)

Binstock, L. 1960. Photographic studies of erythrocytes in ultrasonic fields. M. S. Thesis, Pennsylvania State University.

Bradley, D. E., and Williams, D. J. 1957. An electron microscope study of the spores of some species of the genus Bacillus using carbon replicas. Journal of General Microbiology 17, 75-79.

Brown, I. N. 1969. Immunological aspects of malaria infections. Advances in Immunology 11, 267-349.

Carlin, B. 1964. The use of high- and low-amplitude ultrasonic waves for inspection and processing. "Physical Acoustics" (W. P. Mason, ed.), Vol. 1, pp. 1-55. Academic Press, Inc., New York.

Chambers, L. A., and Gaines, N. 1932. Some effects of intense audible sound on living organisms and cells. Journal of Cellular and Comparative Physiology 1, 451-471.

Christophers, S. R., and Fulton, J. D. 1939. Experiments with isolated malaria parasites (Plasmodium knowlesi) free from red cells. Annuals Tropical Medicine and Parasitolcgy 33, 161.

Clark, J. M. 1964. "Experimental Biochemistry," p. 95. Freeman and Co., San Francisco.

Coggeshall, L. T., and Eaton, M. D. 1938. The complement fixation reaction in monkey malaria. Journal of Experimental Medicine 67, 871-882.

Cook, R. T., Aikawa, M., Rock, R. C., Little, W., and Sprinz, H. 1969. The isolation and fractionation of Plasmodium knowlesi. Military Medicine 134, 866-883.

Cook, R. T., and Aikawa, M. 1971. Malaria resistance: Artificial induction with a partially purified plasmodial fraction. Science 171, 1175-1176.

D'Antonio, L. E., von Doenhoff, A. E., and Fife, E. H. 1966a. A new method for isolation and fractionation of complement fixing antigens from Plasmodium knowlesi. Proceedings, Society for Experimental Biology and Medicine 123, 30-34.

D'Antonio, L. E., von Doenhoff, A. E., and Fife, E. H. 1966b. Serological evaluation of the specificity and sensitivity of purified malarial antigens prepared by a new method. Military Medicine 131, 1152-1156.

D'Antonio, L. E., and Silverman, P. 1971. Malaria resistance: Artificial induction with a partially purified plasmodial fraction. Science 171, 1176.

Dulaney, A. D., and Morrison, D. B. 1944. The preparation and properties of antigens from Plasmodium knowlesi. American Journal of Tropical Medicine 24, 323.

El'piner, I. E. 1964. "Ultrasound: Physical, Chemical, and Biological Effects," pp. 1-25. Consultants Bureau Enterprises, Inc., New York.

Fife, Jr., E. H. 1970. The current state of serological tests used to detect blood parasite infections. Pathogenesis and Immunology of Plasmodial, Babesial, and Trypanosomal Infections, A Symposium Convened at The Ohio State University, June 2 and 3, 1970.

Fife, E. H. 1971. Walter Reed Army Hospital (Personal Communication).

Flynn, H. G. 1964. Physics of acoustic cavitation in liquids. "Physical Acoustics" (W. P. Mason, ed.), Vol. 1, pp. 57-172. Academic Press, Inc., New York.

Frederick, J. R. 1965. "Ultrasonic Engineering," pp. 118-130. John Wiley and Sons, Inc., New York.

Grabar, P. 1953. Biological actions of ultrasonic waves. "Advances in Biological and Medical Physics," pp. 191-246. Academic Press, Inc., New York.

Hughes, D. E., and Nyborg, W. L. 1962. Cell disruption by ultrasound. Science 138, 108-114.

Johnson, C. H. 1929. The lethal effects of ultrasonic radiation. Journal of Physiology 67, 356-359.

Kent, J. F., and Fife, E. H. 1963. Precise standardization of reagents for complement fixation. The American Journal of Tropical Medicine and Hygiene 12, 103-116.

Kirk, R. G. 1968. Interfacial reaggregation of ultrasonically disrupted erythrocyte membranes. Proceedings: National Academy of Science 60, 614-621.

Kreier, J. P., Pearson, G. L., and Stilwill, D. 1965. A capillary agglutination test using Plasmodium gallinaceum parasite freed from erythrocytes. A preliminary report. American Journal of Tropical Medicine and Hygiene 14, 529-532.

Kreier, J. P., Shapiro, H., Dilley, D. A., Sylvassy, I. P., and Ristic, M. 1966. Autoimmune reactions in rats with Plasmodium berghei infections. Experimental Parasitology 19, 155-162.

Kreier, J. P. 1969. Mechanisms of erythrocyte destruction in chickens infected with Plasmodium gallinaceum. Military Medicine 134, 1203-1219.

Ladda, R. L. 1969. New insights into the fine structure of rodent malarial parasites. Military Medicine 134, 825-865.

LeCover, M., and Warner, N. E. 1958. A rapid method for acridine orange fluorescence. American Journal of Clinical Pathology 29, 186-187.

Lehmann, J. E. 1953. The biophysical mode of action of biologic and therapeutic ultrasonic reactions. Journal of the Acoustical Society of America 25, 17-25.

Lombard, D. B. 1955. Ultrasonic rupture of erythrocytes. M. S. Thesis, Pennsylvania State University.

Luft, J. 1961. Improvements in epoxy resin embedding methods. Journal of Biophysical and Biochemical Cytology 8, 409.

Mahoney, D. F., Redington, B. C., and Schoenbechler, M. J. 1966. The preparation and serologic activity of plasmodial fractions. Military Medicine 131, 1141-1151.

Martin, W. J., Finerty, J., and Rosenthal, A. 1971. Isolation of Plasmodium berghei (malaria) parasites by ammonium chloride lysis of infected erythrocytes. Nature 233, 260-261.

Moulder, J. W. 1962. "The Biochemistry of Intracellular Parasitism," pp. 33-41. University of Chicago Press.

Orians, G. H., and Pfeiffer, E. E. 1970. Ecological effects of the war in Vietnam. Science 168, 544-554.

Pautrizel, R., and Nguyen, V. N. 1953. Mise en evidence d'anticorps chez le rat parasite' par P. berghei, a l'aide d'un antigene prepare avec du sang de rat impalude. Bulletin Society of Pathology Exot. 46, 671-673.

Prior, R. B. 1968. The effects of cavitating ultrasonic fields on Plasmodium gallinaceum-infected chicken erythrocytes. M. S. Thesis, The Ohio State University.

Rayleigh, L. 1917. On the pressure developed in a liquid during the collapse of a spherical cavity. Philosophical Magazine 34, 94-98.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology 17, 208-212.

Ristic, M., and Kreier, J. P. 1964. The fine structure of the erythrocytic forms of Plasmodium gallinaceum as revealed by electron microscopy. American Journal of Tropical Medicine and Hygiene 13, 509-514.

Rosenberg, S. A., and McIntosh, J. R. 1968. Erythrocyte membranes: Effects of sonication. Biochimica et Biophysica Acta 163, 285-289.

Rudzinska, M. A., Trager, W., and Bray, R. S. 1965. Pinocytolic uptake and the digestion of hemoglobin in malaria parasites. The Journal of Protozoology 12, 563-576.

Rutledge, L. C., and Ward, R. A. 1967. Effects of ultrasound on Plasmodium gallinaceum-infected chick blood. Experimental Parasitology 20, 167-176.

Schalm, O. W. 1961. "Veterinary Hematology," pp. 70-73. Lea and Febiger Co., Philadelphia, Pa.

Seed, T. M., and Kreier, J. P. 1969. Autoimmune reaction in chickens with Plasmodium gallinaceum infection: The isolation and characterization of a lipid from trypsinized erythrocytes which reacts with serum from acutely infected chickens. Military Medicine 134, 1220-1227.

Smith, F. D. 1935. On the destructive mechanical effects of the gas-bubbles liberated by the passage of intense sound through a liquid. Philosophical Magazine 19, 1147-1151.

Stein, B., and Desowitz, R. S., 1964. The measurement of antibody in human malaria by a formolized tanned sheep cell haemagglutination test. Bulletin World Health Organization 30, 45-49.

Trigg, P. I., Brown, I. N. Gutteridge, W. E., Hockley, D. J., and Williamson, J. 1970. The preparation of free malaria parasites by nitrogen cavitation. Transactions, Royal Society of Tropical Medicine and Hygiene 64, 2-3.

Trubowitz, S., and Masek, B. 1968. Plasmodium falciparum: Phagocytosis by polymorphonuclear leukocytes. Science 162, 273-274.

Verain, A., and Verain, A. 1956. Influence des ultrasons sur Plasmodium berghei. Comptes Rendus des Seances de La Societe de Biologie et de ses Filiales 150, 1189-90.

Weiss, B. 1967. Effects of cavitating ultrasonic fields on the hemolysis and breakdown of Plasmodium gallinaceum-infected chicken red blood cells and the release of erythrocytes--free malaria parasites. M. S. Thesis, The Ohio State University.

Wood, W., and Loomis, A. L. 1927. The physical and biological effects of high frequency soundwaves of great intensity. Philosophical Magazine 4, 417-436.