2. Piez, K. A., Oyama, V. I., Levintow, L., Eagle, H., Nature, 1960, v188, 59.

3. Spackman, D. H., Amino Acid Analyzer Manual, 1962, Spinco Division, Beckman Instruments, Stanford Industrial Park, Palo Alto, Calif.

4. Waymouth, C., J. Nat. Cancer Inst., 1959, v22, 1003.

5. Paul, J., Cell and Tissue Culture, Williams & Wilkins Co., 3rd ed., 1965, 86.

6. Gwatkin, R. B., Siminovitch, L., Proc. Soc. Exp. Biol. and Med., 1960, v103, 718.

7. Paul, J., ibid., 1960, v103, 228.

8. Eaton, M. D., Scala, A. R., Proc. Soc. Exp. Biol. and Med., 1961, v108, 766.

9. Eaton, M. D., Low, I. E., Scala, A. R., Uretsky, S., Virology, 1962, v18, 102.

10. Furusawa, E., Cutting, W., Proc. Soc. Exp. Biol. and Med., 1962, v111, 71.

11. Siegel, S., Nonparametric Statistics, McGraw-Hill Book Co., Inc., New York 1956.

Received March 29, 1966. P.S.E.B.M., 1966, v123.

A New Method for Isolation and Fractionation of Complement Fixing Antigens from *Plasmodium knowlesi.** (31394)

LAWRENCE E. D'ANTONIO, ALBERT E. VON DOENHOFF, JR. AND EARL H. FIFE, JR.
Department of Serology, Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, Washington, D.C.

Although development of methods for effective separation of plasmodia from infected erythrocytes and isolation of purified parasite antigens have been the subject of many investigations during the past 50 years(1), certain major problems have persisted. A variety of methods, including use of enzymes, immune hemolysis, saponin, or other chemical agents, have been employed in efforts to free plasmodia from host red cells(2). Although these agents effectively mediated rupture of the erythrocytes, complete separation of the parasites from host blood components rarely was achieved(3). Moreover, questions have been raised concerning the possibility of a concomitant chemical alteration or loss of antigenic components of the parasite(1,4). Nonchemical lytic methods such as hypotonic lysis, mechanical grinding or cryolysis likewise have been of limited value. These latter methods often resulted in marked destruction of the parasite, or failed to alter the red cell membrane sufficiently to allow effective separation from the parasite. In addition, antigens obtained from parasites processed by these methods frequently exhibited a low order of specificity and sensitivity(1), and in our experience often were anticomplementary.

*Contribution number 78 from the Army Research Program on Malaria.

Preliminary observations on the properties of host red cells and parasites in malaria infections revealed that the mechanical fragility of nonparasitized and parasitized erythrocytes was considerably greater than that of the parasites. The parasites appeared to be quite stable to many of the forces causing rupture of the host red cells. As a result of these observations, it was felt that the precisely controlled conditions obtainable with a French pressure cell(5) (American Instrument Co., Inc., Silver Spring, Md.) might provide a means whereby the host cells could be selectively fragmented without significant alteration of the parasite. The present studies revealed that preferential fragmentation could be achieved if the pressure was carefully regulated. The method which eventually evolved effectively destroyed the red cell membrane with little or no alteration of parasite morphology, and overcame the need for use of lytic agents which could alter or remove parasite antigens.

Materials and methods. Collection of parasitized cells. Rhesus monkeys were infected by intravenous transfer of fresh *Plasmodium knowlesi* infected blood. Upon reaching the terminal cycle, at which time parasitemias usually ranged from 25-30% or greater, the animals were exsanguinated by cardiac puncture. The blood was collected either in modi-

fied Alsever's solution (neutralized to pH 7.0) or heparin and immediately centrifuged in the cold (3°C) at 3500 × *g*. The plasma and buffy coat were removed by aspiration and the cells were resuspended in 0.15 M NaCl solution and recentrifuged. Three additional washings were employed to assure removal of plasma components.

Isolation of parasites and preparation of antigens. Method A. Other than employing veronal rather than phosphate buffered saline, the procedure was that described by Dulaney (6) who employed hypotonic lysis for release of plasmodia from the erythrocytes. The packed, washed erythrocytes were lysed in 10 volumes of distilled water and immediately centrifuged in the cold (3°C) at 2000 × *g*. The supernatant hemoglobin solution was removed by aspiration and the sediment then was washed 3 times in veronal buffered saline, pH 8.6 (VBS). Following each wash, the fluffy brown layer consisting primarily of red cell stromata was aspirated from the packed parasites. The parasite sediment was suspended in 4 volumes of VBS, ground for 3 minutes in a mechanically driven Teflon-glass tissue grinder, and allowed to extract in the cold (3°C) for 2 hours with constant agitation. The mixture then was centrifuged for 10 minutes at 2000 × *g* and the cloudy, dark brown supernatant antigen transferred to a clean test tube. The sediment was extracted again with 4 volumes of VBS and the supernatant solution combined with the initial antigen extract. The pooled extracts were designated Antigen A.

Method B. Plasmodia were released from the red cells by using carefully controlled pressures to selectively fragment the erythrocyte cell walls. The washed cells were resuspended in a volume of saline sufficient to give a 20% suspension, then placed in a cooled (3°C) French pressure cell and slowly forced through the needle valve at a pressure of 1500-2000 psi. The first 2-3 ml were discarded to avoid contamination with the few unruptured erythrocytes which passed through the valve with the initial effluent.

The effluent obtained by the above procedure was centrifuged at 50 × *g* for 10 minutes to remove gross debris. The supernate containing the intact parasites then was centrifuged at 3500 × *g* for 5 minutes at 3°C. The dark brown sediment was resuspended in saline and centrifuged as before. The washing process was repeated 4 times. The washed parasites were suspended in 7 volumes of saline, placed in the French pressure cell, and fragmented by slowly passing through the needle valve at a pressure of 18,000 to 20,000 psi. The effluent then was centrifuged at 10,000 × *g* for 30 minutes to sediment the pigment granules and extraneous material. The clear, light brown supernatant solution constituted Antigen B.

A portion of the B antigen was fractionated by passage through a Sephadex G-200 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) gel filtration column (20 cm height; 2.5 cm dia.) using 0.15 M NaCl solution. An 11-ml portion of the antigen was passed through the column and the effluent was collected in 32 four-ml aliquants. Each fraction was examined spectrophotometrically at wavelengths 2600 and 2800 Å (Beckman Model DU spectrophotometer) and tested in a complement fixation procedure to determine the location of the complement fixing components. The fractions exhibiting maximum complement fixing activity were combined and designated Antigen C.

Serological characterization of antigens. The serological characteristics of the antigens were compared in complement fixation tests wherein serial dilutions of antigen were tested with serially diluted antiserum from a monkey with chronic *P. knowlesi* infection. The antigen controls in the absence of monkey serum provided a basis for comparing the anticomplementary properties of the respective antigen preparations. In addition, parallel complement fixation tests with rabbit anti-Rhesus monkey erythrocyte serum were used to detect possible host red cell contamination in the various antigens.

Results. Separation of plasmodia from erythrocytes by differential fragmentation under the prescribed controlled pressures yielded a product which appeared to consist almost entirely of morphologically intact parasites. No red cell membranes were observed; occasional parasite pigment granules and a

TABLE I. Comparative Titrations of *P. knowlesi* Complement Fixing Antigens.

Antigen	Dilution, 1:	Complement fixation reactions* with *P. knowlesi* monkey antiserum diluted, 1: 4	8	16	32	64	128	Anti-stromata antiserum†	Antigen controls
Antigen A (hypotonic lysis method)	1	4	4	4	4	4	4	4	4
	2	4	4	4	4	4	4	4	4
	4	4	4	4	4	3	3	4	3
	8	4	4	4	4	3	2	4	2
	16	4	3	3	2	1	1	4	1
	32	3	—	—	—	—	—	4	—
Antigen B (unfractionated French press method)	1	4	4	4	4	3	1	1	—
	2	4	4	4	4	3	1	1	—
	4	4	4	4	4	3	±	±	—
	8	4	4	4	4	2	±	—	—
	16	4	4	3	3	1	—	—	—
	32	4	3	3	2	—	—	—	—
Antigen C (Sephadex-fractionated French press method)	1	4	4	4	2	—	—	—	—
	2	4	4	4	2	—	—	—	—
	4	4	4	4	2	—	—	—	—
	8	4	4	3	±	—	—	—	—
	16	4	3	±	—	—	—	—	—
	32	3	3	1	±	—	—	—	—
Serum controls		—	—	—	—	—	—	—	—

* The numerical values represent degree of complement fixation, the negative sign (—) no fixation.

† Serum from a rabbit immunized with monkey erythrocyte stromata.

minimum amount of fibrous-like debris were the only extraneous materials detected. With specimens containing predominantly mature plasmodia, the majority of freed parasites retained their morphological integrity and stained normally. These findings were in marked contrast to those obtained with the older method employing hypotonic lysis for releasing the parasites from the erythrocytes. With the latter, the parasites were badly distorted and stained poorly. In addition, numerous red cell "ghosts" were present and the amount of debris was significantly greater than that encountered with the new separation method.

Serological characteristics of antigens prepared from plasmodia obtained by the two separation methods were compared. The findings are presented in Table I. Examination of the antigen controls revealed that antigen obtained by the older method (Antigen A) was highly anticomplementary. Complete inhibition of complement activity was observed with the higher concentrations (1:1 and 1:2 dilutions) of antigen and some degree of inhibition persisted through the 1:16 dilution. There was also serological evidence of considerable contamination with erythrocyte components; the 1:32 dilution of antigen gave complete fixation with the anti-stromata antiserum. These findings differed significantly from those obtained with antigens prepared by the new procedure (Antigens B & C). Both of the latter were completely free from anticomplementary activity, even when tested undiluted. The weak reactions obtained with the higher concentrations of unfractionated Antigen B in tests with the anti-stromata antiserum indicated the presence of some erythrocyte components. However, the amount of contaminating red cell material was significantly less than that contained in Antigen A and these undesired components were effectively removed by fractionation through the Sephadex gel column (Antigen C). Antigens prepared by the new method reacted well in complement fixation tests with homologous antiserum. On the other hand, critical appraisal of the specific reactivity of Antigen A was not possible because the high level of anticomplementary activity necessitated dilution beyond the range suitable for serologic tests.

A portion of the unfractionated plasmodial

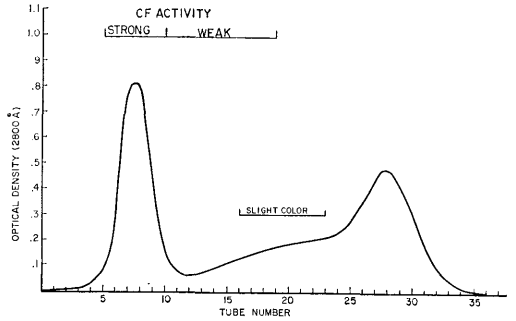

FIG. 1. Sephadex G-200 gel filtration pattern of solubilized *P. knowlesi* constituents. The highly reactive complement fixing (CF) antigens are associated with the first major peak.

antigen (Antigen B) was passed through a gel filtration column in an effort to isolate the desired complement fixing antigen components. Spectrophotometric and serologic evaluations of the various fractions collected from the column indicated that separation of a variety of discrete components was effected. A plot of the relative optical densities of the fractions at wavelength 2800 Å (Fig. 1) showed a major absorbance peak associated with the early fractions collected from the column, a series of minor peaks obtained with the middle fractions, and a final major peak related to the terminal fractions. Tests with *P. knowlesi* antisera obtained from chronically infected monkeys revealed that the first major peak contained the most reactive complement fixing antigens. The succeeding fractions also showed some serologic activity, but at considerably lower levels. The final major peak was serologically inactive. A small number of fractions obtained midway between the initial and final peaks contained essentially all of the pigment of the unfractionated antigen. Moreover, the components responsible for the weak reactions with anti-stromata antiserum resided in a fraction (Tube 12, Fig. 1) that was distinct from those containing the major complement fixing antigens. Spectrophotometric analyses of the various fractions at wavelength 2600 Å yielded absorption peaks which in general coincided with those observed at 2800 Å.

Discussion. Antigen prepared by a procedure using hypotonic lysis for release of the parasites from the erythrocytes(6) illustrated some of the problems associated with earlier methods for obtaining malaria antigens and provided a basis for comparing the efficacy of the new procedure employing the French pressure cell. Results of the present studies indicated that many of the previous problems could be overcome by preferential fragmentation of the parasitized blood, provided that the pressure was carefully controlled (1500-2000 psi) and that a well fitted needle valve was employed. In contrast to the product obtained by hypotonic lysis, passage through the pressure cell appeared to completely destroy the red cell membrane without apparent alteration of the parasites. Stained smears of the effluent from the pressure cell revealed only intact parasites and a small amount of amorphous debris. The red cell membranes apparently were disintegrated to the extent that the fragments remained in suspension during centrifugation at $3500 \times g$ for 5 minutes and thus could be readily separated from the intact parasites. Final rupture of the parasites under high pressure (20,000 psi) completely disintegrated all cellular material and the sediment obtained by centrifugation of the effluent consisted almost entirely of granular elements, presumably chromatin.

The unfractionated antigen (Antigen B) prepared by the new procedure was superior in many respects to the product (Antigen A) obtained by the earlier method (*cf* Table I). Antigen prepared by the new method was highly reactive in complement fixation tests with homologous antiserum, showed no evidence of anticomplementary activity even when tested at high concentration, and appeared to contain only trace amounts of erythrocyte contaminants. Although the latter did not appear to seriously limit use of the antigen in serodiagnostic tests, the problem was overcome by fractionation through a gel filtration column. Passage of the solubilized parasite material (Antigen B) through a Sephadex G-200 gel column effectively separated the major complement fixing antigen from the other components. The antigen fraction (Antigen C) was colorless, reacted well with homologous antiserum, and was not anticomplementary at any concentration tested. Furthermore, complement fixation

tests with rabbit anti-Rhesus monkey erythrocyte serum revealed that the major antigen fraction was free from detectable red cell contaminants. The trace amounts of these undesirable components that were observed in the unfractionated Antigen B resided in a fraction distinct from those containing the major complement fixing antigens. In this regard it is noteworthy that the complement fixation test is considerably more sensitive than the precipitation technics often employed for detection of contaminants and thus provided an especially critical method for appraising the purity of the respective antigen preparations.

Preliminary studies on the physicochemical properties of the major complement fixing antigen fraction revealed that the antigen was highly stable. The fractionated material showed no reduction in complement fixing activity following storage for at least 2 months at 4°C. In addition, there was no evidence of deterioration as a result of freezing (—70°C). Stability of the antigen was not altered by heating at 56°C for 1 hour. However, heating at 100°C for 5 minutes resulted in some loss of serologic activity. Addition of polyvinylpyrollidone (1%) permitted lyophilization without loss of activity.

The technics described herein have overcome certain major problems inherent in the methods previously employed for obtaining purified plasmodial antigens. The present technics appear to have immediate value in facilitating precise analytical studies on the composition of the malaria parasite and for the isolation and identification of components involved in host-parasite relationships. In addition, it is anticipated that the procedures can be advantageously used as a model for fractionation of other species of *Plasmodium* and possibly other red cell parasites such as *Babesia* and *Anaplasma*. In this regard it is noteworthy that recent preliminary studies have shown that the new technics can be used for production of highly reactive complement fixing antigens from *P. berghei* and *P. falciparum* as well as *P. knowlesi*. Comprehensive serological evaluations of these antigens currently are in progress.

Summary. A new method is presented for nonchemical isolation and fractionation of the malaria parasite *Plasmodium knowlesi*. Since the mechanical fragility of erythrocytes is considerably greater than that of the plasmodia, it was possible to preferentially fragment the red cell membrane without apparent alteration of the parasites. This was achieved by utilizing the precisely controlled conditions obtained with a French pressure cell operated at 1500-2000 psi. Parasites isolated by this method were subsequently fragmented at higher pressures (20,000 psi) and the solubilized substances fractionated by gel filtration. Fractions showing the major complement fixing activity reacted strongly with *P. knowlesi* antisera, were colorless, exhibited no anticomplementary properties and showed no serologic evidence of red cell contaminants. The findings indicate that the method may provide essential material for further studies on plasmodial composition and be of particular value for isolation of other intra-erythrocytic parasites.

Grateful acknowledgment is made to Dr. D. T. O. Wong for his valuable suggestions and to Miss Jennie Ciak and Mr. John Alenick for assistance in use of the French press.

1. Tobie, J. E., Am. J. Trop. Med. & Hyg., 1964, v13, 195.

2. Zuckerman, A., ibid., 1964, v13, 209.

3. Sherman, I., ibid., 1964, v13, 214.

4. Diggs, C. L., ibid., 1964, v13, 217.

5. Ribi, E., Perrine, T., List, R., Brown, W., Boode, G., Proc. Soc. Exp. Biol. and Med., 1959, v100, 647.

6. Dulaney, A. D., Morrison, D. B., Am. J. Trop. Med., 1944, v24, 323.

Received April 11, 1966. P.S.E.B.M., 1966, v123.