December 4, 1944

To the Committee on Graduate Study:

I am submitting to you a thesis written by Grover W. Austin entitled "Studies on Lophurae Antigen for use in Complement Fixation in Malaria". I recommend that it be accepted for nine quarter hours credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Bacteriology.

Anna Dean Delaney
Major Professor

We have read this thesis
and recommend its acceptance:

Douglas H. Sprunt

I. D. Michelson.

Accepted for the Committee

T. C. Smith
Dean of the Graduate School

STUDIES ON LOPHURAE ANTIGEN FOR USE IN COMPLEMENT FIXATION IN MALARIA

A THESIS

Submitted to
The Committee on Graduate Study
Of
The University of Tennessee
in
Partial Fulfillment of the Requirements
for the degree of
Master of Science

---

by

Grover W. Austin

December 1944

TABLE OF CONTENTS

	PAGE
INTRODUCTION	1
STATEMENT OF PROBLEM	2
REVIEW OF LITERATURE	2
MATERIALS AND METHODS	32
EXPERIMENTAL	35
Experiment 1	36
Experiment 2	38
Experiment 3	40
Experiment 4	44
Experiment 5	48
Experiment 6	53
Experiment 7	55
Experiment 8	61
Experiment 9	64
Experiment 10	69
SUMMARY AND CONCLUSIONS	75
BIBLIOGRAPHY	82

12 Mr45 SLB 1.25

204066

LIST OF TABLES

TABLE		PAGE
I.	Showing the Relative Activity of Extracts of *P. lophurae* Parasites.....	43
II.	Comparison of the Activity of Saline Extracts of Parasitized Cells Laked with Saponin and with Water.....	46
III.	Activity of Barbiturate Buffer Extracts of Parasites Treated with and without Incubation	52
IV.	Comparison of Barbiturate Buffer Extracts and Phosphate Buffer Extracts of Wet Parasites.....	54
V.	Showing the Activity of Saline Extracts of Dried *P. lophurae* Parasites.....	59
VI.	Showing the Antigenic Activity of a $NaHCO_3$-HCl Extract of Dried *P. lophurae* Parasites.....	63
VII.	Comparison of Barbiturate Buffer Extracts and Phosphate Buffer Extracts of Dried Parasites.....	67
VIII.	Activity of Phosphate Buffer Extracts of Citrate-Dextrose Treated Parasitized Cells.....	73

ACKNOWLEDGEMENT

This work was partly subsidized by a grant from the Tennessee Valley Authority through the Department of Preventive Medicine, University of Tennessee.

I wish to express my appreciation to Dr. A. D. Dulaney and to Dr. D. B. Morrison for their helpful suggestions and counsel and to Miss Frances Babbin for her technical assistance.

STUDIES ON LOPHURAE ANTIGEN FOR USE IN COMPLEMENT FIXATION IN MALARIA

Serological studies in malaria have constituted the chief subject of research in this laboratory for the past 5 years. Of the various tests complement fixation has proved most successful.

Antigens prepared from blood of monkeys infected with P. knowlesi have been extensively used for tests with human sera. Monkeys, however, are expensive, hard to handle, and at times, difficult to obtain.

Ducks have been the hosts for extensive investigations on P. lophurae malaria by other workers (9, 10, 11) at this school and were therefore available to us. Since the malaria antigen is group specific, we believed it worthwhile to attempt the preparation of antigen from P. Lophurae.

An average-sized duck weighing from 2 to 3 kilograms produces approximately as much blood as can be obtained from a good-sized monkey. In addition, the blood of ducks is more heavily parasitized than is that of monkeys. Ducks may survive parasitemias of 100-150% with mature schizonts or presegmenters, whereas the monkey seldom survives a segmentation beyond the stage of 40-50% parasitemia. Lastly, one fact to be remembered is the greater simplicity and the shorter time consumed in bleeding ducks.

In this problem, we have concerned ourselves chiefly with the methods of preparing P. lophurae antigens for use in malaria complement fixation tests. The problem of specificity is now in the process of study and investigation in our laboratory, and is not dealt with here.

A. Malarial Antigens from Parasite Cultures

There are many reports in the literature of attempts to prepare malarial complement fixation antigens. A composite presentation follows:

Thomson (29), in 1918, described the preparation of a specific malarial antigen from cultures of malaria parasites. The blood for culture was obtained from a heavily infected patient and incubated for 24 to 48 hours. The supernatant serum was then pipetted off, and distilled water added in excess to the remaining red blood cells. The cells were shaken and centrifuged and the supernatant fluid removed each time. This process was repeated until the fluid was free of hemoglobin. The sediment at the bottom consisted of parasites, envelopes of red blood cells, and white blood cells. The sediment was dissolved in a small quantity of N/10 NaOH which resulted in a straw-colored fluid. This was neutralized by adding N HCl drop by drop. This solution was diluted ten times with normal saline and tested for anticomplementary

properties by titrating with varying dilutions of guinea pig serum. This was carried out in ascending dilutions until an antigen was obtained which did not of itself prevent the action of complement. The best antigen in this study was demonstrated when the parasite material was diluted 1:30 with normal saline.

For the test, the patient's serum was diluted 1:30 with normal saline and standard doses added to 4 tubes, 3 of which contained antigen. The fourth served as the serum control. Into the 3 tubes containing antigen were introduced 2½, 3, and 3½ doses of complement, and 2½ doses were added to the serum control. An antigen control was run with each series. Overnight icebox fixation was used. On the following morning, sensitized red blood cells were introduced, and the results read after water-bath incubation at 37° for 15 minutes.

There were studies made of 200 known cases of benign and malignant tertian malaria with or without parasites in the peripheral blood, and at all stages of quinine treatment. Complement fixation was irregularly demonstrated.

The author states that the anticomplementary properties of this antigen varied greatly, it being necessary at times to dilute the original antigen 100 times before it could be used, which obviously decreased the sensitivity of the test. It was further found that this antigen reacts with

sera of syphilitic patients. Hence, it is necessary to exclude this disease from sera tested with this antigen.

Thomson (30), in the following year, conducted a series of experiments for the purpose of determining if a specific antigen could be prepared for benign and malignant agnes. The antigen was prepared from malaria parasites as described above.

The serum of patients with P. falciparum infection reacted with the P. falciparum antigen, but were negative to the P. vivax antigen. One patient with benign tertian was positive to both. Four P. vivax and 2 P. falciparum sera were positive to a spleen extract of P. falciparum. All except 1 P. falciparum serum were positive to a benign tertian antigen. Twenty-one of 22 P. vivax and 5 P. falciparum sera gave positive responses to an antigen composed of 10 strains of P. vivax. Nineteen patients whose blood contained P. vivax were tested; 11 gave a strong positive, 7 a weak positive, and 1 a negative reaction.

B. Malarial Antigens from Oocysts in Infected Anophelines

Manson-Bahr (22), in a discussion of Thomsin's work on attempts to prepare a specific antigen from parasite cultures, suggested that a strong and specific antigen might

possibly be obtained from the oocysts in infected anophelines in the same way as Fairley (7) had extracted a powerful antigen for bilharzia from the livers of snails harboring the cercariae of human schistosomes. In 1920, Manson-Bahr attempted to obtain a specific antigen from oocysts of plasmodia found in the stomachs of A. maculipennis. With the aid of James and Gill, he obtained, with great difficulty, an antigen extract from the stomachs of 26 anophelines. He next collected the oocysts of the bird malaria parasite from 24 infected Culex fatigens. With this material he could not demonstrate complement fixation. He believed that this procedure would be successful if at least 100 stomachs per cc. of absolute alcohol could be used.

C. Malarial Antigens from Parasitized Human Blood and Tissues

Kingsbury (14), in 1927, attempted to prepare a specific antigen for benign and malignant malaria from parasitized human red blood cells. This series of experiments included the testing of 11 antigens--8 from the spleen, liver, heart, brain, and blood of a subtertian infected patient who died comatosed after a single quinine injection; and 3 from the blood of a patient 1/16 of whose red blood cells were parasitized with P. vivax. The most satisfactory

antigens were saline emulsions of washed and lysed blood. The results are as follows:

1. Twenty-five subtertian malaria sera reacted with P. falciparum antigen, and 48% were positive.
2. Sixteen subtertian sera were tested with P. vivax and 31% read positive.
3. Twelve tertian malarial sera were tested with P. falciparum antigen with 50% reading positive.
4. Six tertian sera were tested with P. vivax antigen and 67% were positive.

Massa (23), in 1929, prepared a P. falciparum antigen from a placenta of a woman who had recently experienced a malarial access. The method of preparing such an antigen was not reported. He does state, whoever, that complement deviation occurred in half of 25 cases held to be malarial. It was positive in 4 of 8 cases who did not show plasmodia, and negative in "primitive" malaria, and in those showing gametes only. The action was not specific and was intensified by preliminary eliminations of hemolytic amboceptor. There was no parallelism between the intensity of the reaction and the clinical importance of infection.

D. Malarial Antigens from Parasitized Chicken Blood and Tissues

Kligler and Yolei (15), in 1941, report studies of antigens made from P. gallinaceum. Extracts were prepared

from blood, liver, and spleen by extracting the dried materials with saline by freezing and thawing. One antigen was prepared from infected blood by first laking with distilled water and then extracting the sedimented parasites with saline by freezing and thawing. The blood antigen gave positive reactions in dilutions up to 1:800 with hyperimmune monkey serum as well as with immune chicken serum.

The P. gallinaceum antigen reacted in a higher dilution with monkey immune serum than with homologous chicken serum, due probably, to the greater antibody content of the monkey serum. Normal chicken sera gave negative reactions with the P. gallinaceum antigen.

They next tested the P. gallinaceum antigen with sera from human subjects both with and without malaria. For comparison and control, the sera from malaria patients were also tested simultaneously with P. knowlesi antigen.

It was found that the P. gallinaceum antigen in a dilution of 1:400 gave positive complement fixation reactions with those sera from malaria patients which gave a positive reaction with the P. knowlesi antigen.

As a control, the authors used an antigen prepared from red blood cells of normal chickens. The antigen was

prepared in exactly the same manner as the plasmodial antigen. At times this antigen fixed complement with sera from malaria patients, although it failed to react with normal human sera or with those thus far tested from other patients (lues, typhoid, infective jaundice). The nature of this reaction is now being elucidated.

The authors are of the opinion that P. gallinaceum antigen may fulfill the same function as the P. knowlesi antigen, but further work must be carried out before definite conclusions can be established.

E. Malarial Antigens from Parasitized Monkey Blood and Spleen

Eaton and Coggeshall (6), in 1938, describe the preparation of 4 malarial antigens, two from the blood and two from the spleen of monkeys dying from P. knowlesi infections.

The red blood cells of the parasitized monkey were concentrated, frozen, dried and preserved in sealed tubes. The spleens were dried in a similar manner.

Antigen 1.-- prepared from parasitized red blood cells by rehydrating the dried equivalent of 1 cc. of packed cells with 10 cc. of saline. The resulting suspension was frozen and thawed four times, centrifuged, and the supernatant used as antigen. It was not anticomplementary, and was used in tests in a dilution of 1:4.

Antigen 2.-- prepared from dried parasitized red blood cells ground in a ball mill and extracted with saline. The proportion of dried cells to saline was the same as for antigen 1. This antigen, when undiluted, was slightly anticomplementary, but could be used for tests in a 1:10 dilution.

Antigen 3.-- prepared from dried spleen by a method similar to that used in the preparation of antigen 2, using 1 gram of dried spleen to 10 cc. saline. It was definitely anticomplementary, and was used in tests in a dilution of 1:10.

Antigen 4.-- prepared from dried spleen by rehydrating 1 gram of dried material with 10 cc. saline, and freezing and thawing as in antigen 1. This antigen was anticomplementary in a dilution of 1:2, but was used in tests in a dilution of 1:12.

Antigen N.-- prepared from normal monkey red blood cells with the technique similar to that used in antigen 1. Antigen N was used for a normal control in the tests with malarial sera, and was not anticomplementary even in the undiluted state.

None of these antigens showed hemolytic properties when tested with sensitized sheep cells.

Titrations were carried out with hyperimmune monkey serum. In this reaction, non-specific factors may be

considered negligible. The amount of reactive monkey protein was measured by titrating against the serum of a rabbit immunized with normal monkey red blood cells.

With the antimonkey (rabbit) serum, antigen 4 gave fixation of complement at a dilution which was four times that of antigen 1. With the antimalarial (monkey) serum, antigen 4 was slightly less reactive than antigen 1. This indicates that antigen 4 contained more non-specific material (monkey protein) per reacting unit of malarial antigen than did antigen 1. Furthermore, antigen 4 was more anticomplementary than was antigen 1.

Antigen N reacted to a slightly higher titer with the antimonkey serum than did antigen 1. In the complement fixation tests with human sera, both antigens were used at dilutions of 1:4. The sensitivity of antigen N to substances reactive with the monkey cells should, on the basis of the results with rabbit serum, be slightly greater than the sensitivity of antigen 1. In this way, false-positive reactions due to constituents of the red blood cells could be detected. The authors pointed out that the standardization of malarial antigen and of normal control antigens can be accomplished with reasonable accuracy when the methods just described are used in conjunction with tests against malarial and non-malarial human sera. When a new preparation of antigen is made, this is compared with

the old antigen by parallel tests, and the concentration is then adjusted so that the new antigen gives reactions of similar sensitivity and specificity.

The 4 malarial antigens were tested with normal human sera and with Wassermann positive and Wassermann negative luetic sera. Antigens 1 and 2 gave less frequent and weaker positive reactions than antigens 3 and 4. With 1 and 2, approximately the same percentage of positive and doubtful reactions occurred with luetic as with normal sera. With antigen 3, a larger number of positive reactions was obtained with Wassermann positive than with Wassermann negative luetic sera, and the percentage of positive reactions with normal sera was considerably lower, being about the same as with 1 and 2. These results indicate that antigen 3 contained considerable amounts of substances which fix complement in parallel with the Wassermann reaction. Antigen 4 did not give a greater percentage of positive reactions with Wassermann positive than with Wassermann negative luetic sera, but the proportion of positive reactions with these sera was slightly higher than was given by 1 and 2. It is readily seen that antigen 3 cannot be considered reliable for tests with malarial sera which came from patients suffering from paresis, because of its reactivity with non-malarial luetic sera. The other 3 antigens gave less cross reactions with luetic sera, and

with these antigens a reaction of 2-plus serum in amounts of 0.1 cc. or less was considered significant.

In conjunction with this series of experiments, these workers observed the serological reactions of normal monkey red blood cells with normal, luetic, and malarial human sera. Several investigators have recorded an agglutination of the red blood cells of rhesus monkeys by some human sera. This is apparently due to hetero-agglutinins not related to the human blood groups or to the Forssman antigen. In the present work, some of the patients with P. knowlesi infection had been inoculated with the infected blood of rhesus monkeys, and their sera showed definite agglutination of monkey cells. It was, of course, necessary to determine to what extent these reactions affected the complement fixation with monkey antigens and human sera in the detection of malarial antibodies.

The antigen was prepared and standardized for sensitivity by titrating against anti-monkey (rabbit) serum and used in 1:4 dilution. Of 150 sera tested, only 1 gave a reaction more positive in an amount of 0.1 cc. with the normal monkey antigen. There was no important difference between luetic sera giving a positive Wassermann, those giving a negative Wassermann, and the normal sera. In the 3 series of malarial sera the percentage of positive and

doubtful reactions were considerably higher than with the non-malarial sera. This suggests that malarial infection stimulates the production of hetero-antibodies which react with normal monkey cells. In two or three cases the apparent development of such antibodies at low titer during the course of the malarial paroxysms was observed in patients who had not received monkey blood. These observations demonstrate the importance of using, in complement fixation tests for malaria, a control antigen made from normal cells in the same way and used at the same effective dilutions as the malarial antigen.

On 21 non-malarial sera and 28 malarial sera, agglutination and complement fixation tests with normal monkey cells were run in parallel. Of the total of 49 sera, 28 (14 in each group) gave negative reactions in both tests. With 5 malarial sera and 5 non-malarial sera, the agglutination test was positive and the complement fixation test negative, and with 5 malarial and 1 non-malarial sera both tests were positive. One serum gave weak complement fixation, but no agglutination. These results indicate that with a fair proportion of sera the hetero-antibodies that cause agglutination of normal monkey cells react weakly or not at all in the complement fixation tests. Strong complement fixation reactions due to the antigens in normal monkey cells are rare. Consequently, strong

reactions with a malarial antigen prepared from parasitized red blood cells may be considered significant. When the reaction with malarial antigen is weak or doubtful, the control with normal antigen is of value in eliminating a certain number of false-positives. In general, pseudo-positive reactions were more frequent and stronger with the antigens prepared from spleen than with the antigens prepared from blood. From the results it is evident that the control tests run with antigen N are of value in eliminating about half of the false-positive reactions when parasitized blood is used for the test, but such controls are of little value in eliminating the more frequent false-positive reactions with spleen antigen.

Eaton and Coggeshall conclude that:

1. In the complement fixation test a positive reaction between any serum and a complex material prepared from blood or spleen is a summation of the reactions between several antigens with their corresponding antibodies.
2. Though the malarial antigen appears to be a water-soluble protein, whereas the Wassermann antigen is probably a lipoid, certain preparations may contain both the Wassermann antigen and the malarial antigen. For example, antigen 3, prepared from malarial spleen, gave a much higher

percentage of positive complement fixations with Wassermann positive sera than with Wassermann negative or normal sera.

3. With luetic sera from patients having no malaria, 3 of the 4 malarial antigens tested gave no higher percentage of positive reactions with Wassermann positive than with Wassermann negative sera, and antigen 1 was not more reactive with luetic sera than with normal sera.

4. When the reactions of normal and luetic sera with antigens prepared from parasitized red blood cells (antigen 1) and normal red blood cells (antigen N) are compared, it is found that the malarial antigen gives a slightly higher percentage of positive reactions with both normal and luetic sera, but the difference in reactivity of antigen 1 and antigen N with these sera are not great enough to be significant.

5. Certain human sera contain hetero-agglutinins for normal monkey cells, and these antibodies also give a weak complement reaction with extracts of frozen and thawed monkey red blood cells.

6. These hetero-antibodies are increased during malarial infection, but they never approach the

level of the immune bodies which fix complement with the malarial antigen.

7. Since adsorption of malarial sera with normal monkey red blood cells reduces the complement fixation titer against malarial antigen from parasitized blood only slightly or not at all, it may be concluded that most of the fixation of complement obtained with malarial serum is due to malarial antigen-antibody reactions and not to reactions of hetero-antibodies with constituents of the red blood cells.

8. With the exception of one antigen prepared from the spleen, there was no evidence that the malarial antigens were more reactive with Wassermann positive than with Wassermann negative sera.

9. Some human sera give weak complement fixation with antigens prepared from normal monkey red blood cells, and the percentage of these positive reactions is only slightly higher with malarial sera than with normal or luetic sera.

10. The most sensitive and specific malarial antigen was prepared from dried parasitized red blood cells by extraction with saline and freezing and thawing. This *P. knowlesi* antigen is group

specific in that it gives a strong complement fixation with malaria sera from human beings infected with either *P. knowlesi*, *P. vivax*, or *P. falciparum*.

Coggeshall and Eaton (2), in the same year, reported another series of experiments in which they prepared four other antigens to be used in complement fixation reactions in monkey malaria.

Antigen 1.-- prepared from malarial infected spleens which were chopped up, frozen, and dried in the frozen state in a vacuum desiccator. The dried material was then ground in a ball mill at -70°C. The ground material was extracted overnight in the refrigerator with 10 cc. saline for each gram and the insoluble residue centrifuged down. The supernatant was used as antigen.

Antigen 2. -- prepared from blood containing 20-50% parasitised red blood cells. The blood was collected in 2% sodium citrate solution, centrifuged to separate the serum, and washed two times with saline. The packed red blood cells and parasites suspended in an equal volume of saline were then frozen, dried, and ground by the same procedure as that used for spleens. The antigen was finally prepared by extraction with saline.

Antigen 3. -- prepared from parasitised blood, washed as described above, and mixed with 3 volumes of distilled

water. Toluene was added as a preservative, and the material allowed to autolyze in the incubator for 48 hours. The insoluble residue was centrifuged down and the supernate used as antigen after adding enough saline to bring it to isotonicity.

Antigen 4.-- prepared by adding 3 volumes of distilled water to parasitized blood which had undergone autolysis in the refrigerator for several weeks, and then centrifuging.

From their results, the authors conclude that:

1. The material extracted from infected spleen by acetone, alcohol, or ether does not give a specific fixation of complement with immune serum.
2. The material extracted from spleen or blood with acid or alkaline buffers is not a better antigen for complement fixation than that extracted by saline or distilled water.
3. Since the antigen was not extractable by lipoid solvents and was destroyed by tryptic digestion, the active principle behaves as a protein.

Coggeshall (1), 3 years later, gives an account of preparing an antigen from parasitized red blood cells obtained from rhesus monkeys whose red blood cells were 50% parasitized with P. knowlesi. The red blood cells were

washed free from serum and preserved by freezing and drying in 5 cc. amounts. The stored antigen was rehydrated and diluted 1:100 in normal saline, which is well beyond the anticomplementary range. Titration showed the preparation to be highly antigenic.

It was found that serum of patients with vivax and falciparum malaria would fix complement with the monkey parasite antigen in approximately the same dilutions as serum from patients with P. knowlesi malaria (used in treatment of general paresis).

Stratman-Thomas and Dulaney (2, 8), in 1940, report the use of 4 different antigens for the use of complement fixation tests prepared from: (1) infected and (2) normal monkey blood, (3) infected and (4) normal human blood.

The monkey antigens and normal human antigens were prepared as described by Coggeshall and Eaton (2, 6). The former from monkeys infected with P. knowlesi. The infected human antigens were obtained from patients undergoing induced malarial therapy who had received mosquito inoculations of P. vivax or blood inoculations of P. malariae. They were prepared from parasites collected by centrifugation after laking heavily parasitized blood cells. This parasite mass was dried in vacuo, and ground. When ready for use, 0.1 gram was rehydrated with 10 cc. saline, frozen, thawed, centrifuged, and the supernatant used as antigen.

Ninety-seven individuals were tested with the 4 antigens. This number included 62 patients with established malaria and 35 normals. Fifty of the 62 patients were receiving malaria therapy for paresis. Twelve had naturally acquired malaria. Specimens of blood were drawn at intervals during the course of the disease. The results of this study are as follows:

1. The antigens prepared from blood of injected monkeys and human individuals yielded similar results.
2. A small percentage of sera yielded positive results with normal antigens.
3. Of the 50 patients with induced malaria, only 34 (68%) showed a positive complement fixation reaction.
4. Of the 35 normal controls regarded as uninfected with malaria, 30 (87.5%) were negative.

The authors conclude that:

1. A positive Wassermann does not interfere with the complement fixation test using a malaria antigen, since 25 of the 35 controls gave a positive Wassermann reaction.
2. Antigens prepared from human blood are just as satisfactory for complement fixations as those prepared from monkey blood.

3. The source of the malarial antigen is still debatable. It may not be the malaria parasite itself, but possibly the stroma of the parasitized red blood cell.

Later in the same year, Dulaney and Stratman-Thomas (4) report further studies with various antigens prepared from the blood of infected monkeys and human beings.

In all cases the parasite count and stage of development of the malarial plasmodia were followed, and blood for antigen was obtained at a time when the greatest yield as to number and size of parasites was assured. In human blood the counts ranged from 300-500 parasites per 100 white blood cells with approximately 0.5-0.7% of red blood cells containing parasites. The monkeys were bled when the animals were near death, and blood smears showed a parasitization ranging from 20-40% with a high proportion of mature malarial parasites.

The methods of preparation and value of various antigens tested may be generalized as follows:

1. Saline or watery extracts of washed parasitized human or monkey red blood cells, or of organs from infected monkeys. Inconsistent results were obtained with these preparations.
2. Papain digests of malarial blood clots or parasite masses. These digests gave negative results.

3. Parasite masses recovered from laked cells which were treated with NaOH and then neutralized with HCl. This method did not yield successful antigens.

4. Red blood cells of parasitized human beings and monkeys, which were dried, then rehydrated and the resulting suspension frozen and thawed, centrifuged, and the supernate used as antigen. This was Coggeshall and Eaton's method of preparing monkey-blood antigens which gave highly specific reactions in monkey and human malaria.

5. Parasites from human and monkey bloods which were treated in the same manner as the red blood cells in 4.

The authors state that the antigen of higheat specificity and sensitivity was that prepared from malarial parasites from human or monkey hosts.

Their method of harvesting the parasites is as follows:

1. Collect blood (human or monkey) in physiological saline solution containing 2% sodium citrate.

2. As soon as possible centrifuge, remove plasma and wash the red cells two or three times with sterile saline solution.

3. Lake the red cells with cold distilled water. Distilled water has been found to be the

best reagent for preserving the integrity of the parasite. Collect the parasites by centrifugation. The parasites will be thrown to the bottom of the tube in a viscous mass and stained smears will reveal highly intact plasmodia with some red cell stroma and a very few white blood cells.

4. Wash the parasite-mass free of hemoglobin with water and physiological saline solution.

5. Dry in vacuo. Grind; place in sealed tubes, and store at low temperature.

6. When ready for use, grind 0.1 gram of dried antigen in a mortar with saline solution until 10 ml. have been used. Freeze and thaw four times with a dry ice-alcohol mixture.

7. Centrifuge and standardize the supernate by titration with known positive and negative malarial sera, and test for anticomplementary properties. We have made a practice of pooling parasites from several sources so that one standardization will suffice. In the case of monkey parasites the antigen has most often been used after dilution of the supernate 1:2 and in doses of 0.1-0.2 ml. Thus, 0.1 gram of the dried parasites will prove sufficient for approximately 90-100 tests.

They found the dry antigen to be suitable for use after storage of 6 months. The rehydrated antigen, when kept in the icebox for several weeks, did not lose its potency.

The authors performed complement fixation tests on a large number of sera from patients with induced and natural malaria, with sera giving positive and negative Wassermann reactions, and with sera of individuals pre-

sumably free of malaria whose Wassermann reactions were unknown. Four hundred and forty-two sera were tested from 217 individuals. One hundred and sixty (53%) of the 308 sera from 83 malarial patients gave a positive reaction. Some of these sera were obtained early in the course of the disease before parasites were demonstrated in blood smears, others after quinine-therapy had been instituted. In the control group of 134, only 7 (5%) gave a positive reaction. These were weak reactions, never over 1-plus. Thus a striking difference is apparent between the two groups. Complement fixation with malaria antigen has significance.

These workers offer further substantial evidence to Kingsbury's belief that both the complement-fixing antibody and the malarial antigen are group rather than species-specific. The sera of nine patients inoculated with one of 3 types of malarial parasites (P. knowlesi, P. vivax, or P. malariae) were tested with each of the 3 antigens prepared from the same types of parasites. The authors found that any one of the antigens was suitable for detecting complement-fixing antibodies. They regard the P. knowlesi antigen as the best for practical reasons. This antigen has proved to give as strong, if not stronger, reactions in comparative complement fixation tests using the human malarial parasite antigens.

In the series of patients studied, the authors did not find syphilis to influence the reaction with their malarial parasite antigen. In the group whose Wassermann reactions were known, they did not at any time obtain a higher percentage of non-specific reactions with Wassermann positive than with Wassermann negative sera. They were, however, reluctant to draw any conclusions regarding the much debated question as to whether or not malaria provokes a non-specific Wassermann reaction. They did show that Wassermann patients who acquire malaria may give a negative Wassermann reaction at a time when they have developed a strongly positive reaction for the malarial antigens. Furthermore, it was observed that Wassermann negative patients may give a negative Wassermann reaction and a 4-plus complement fixation for malaria at a time when malarial parasites are easily demonstrated in blood smears.

Dulaney and Morrison (3), in 1944, report on the preparation and properties of various antigens from P. knowlesi.

The following types of parasite products are cited:

1. Saline extracts of dried parasites.
2. Phosphate buffer extracts of wet and dried parasites.
3. Barbiturate buffer extracts of wet and dried parasites.

4. Solutions of parasites obtained by treatment with barbiturate buffer and NaOH.

5. NaOH solutions of parasites.

The preparation of saline extracts as described previously (Dulaney and Stratman-Thomas, 1940) was not altered. It was found that one freezing and thawing released into the extract only a part of the antigenic material. When the residue obtained by centrifugation was taken up in the original volume of saline (10 cc. per 0.1 gram of dried parasites) and again frozen and thawed four times, the resulting supernate had one-fourth to one-half the antigenic activity of the first. They state that third and fourth extracts may be as active as the second. The first extract was a deep amber color, clear and sparkling; the others almost colorless. With 4 successive extractions a calculated total of 1200-1600 antigenic units was obtained from 0.1 gram of dried parasites. With the average yield of 0.5 gram of parasites per monkey it is evident that 6000-8000 units of antigen may be obtained from one animal. It is stated that the parasite yield has reached 0.8-1.0 gram in large and heavily parasitized monkeys.

Antigens have been prepared by treating wet or dried parasites with M/10 phosphate buffer of pH 7.8-8.0. These antigens were 6-8 times more active than the saline extracts.

These authors attempt to compare the relative activity and nitrogen content of various phosphate extracts with those of saline. They proceeded as follows:

Two 0.1 gram samples of dried parasites were each ground with 10 cc. of phosphate buffer and labeled (a) and (b). A third sample was ground with 10 cc. of 0.9 per cent NaCl (c). Preparation (a) was frozen and thawed four times, centrifuged, and the clear brown supernate removed and designated P-FT-1. The residue was mixed with 10 cc. of phosphate buffer and the freezing and thawing process repeated. Supernates P-FT-2, P-FT-3, and P-FT-4, obtained in like manner, were practically colorless.

Preparation (b) was allowed to stand at room temperature with frequent stirrings for 1 hour. Centrifugation yielded a deep brown opalescent supernate which was designated P-R-1. The residue was taken up in 10 cc. of buffer and stirred at intervals during a second extraction period of an hour; the pale amber opalescent supernate was designated P-R-2. Third and fourth extractions yielded faintly colored P-R-3 and P-R-4 preparations. The residue from the fourth extraction was taken up in 10 cc. of buffer and left in the refrigerator over night. This was centrifuged and the supernate designated P-R-5.

Preparation (c) was frozen and thawed as was (a), using 10 cc. amounts of 0.9 per cent NaCl and S-FT-1,

S-FT-2, S-FT-3, and S-FT-4 extracts obtained.

The relative antigenic activity was determined by titration with a strongly "positive" malaria serum diluted 1:2½. A known negative serum and anticomplementary controls were included, all of which gave negative tests. A calculated total of 9600 antigenic units was obtained by 4 phosphate buffer extractions at room temperature, 7200 units by 4 freezings and thawings in phosphate, and 1400 units by 4 freezings and thawings in saline.

Five cc. samples of each of the 13 preparations were used for total nitrogen determinations. The total nitrogen determinations indicated that the first extracts by any method contain more nonantigenic material than subsequent extracts. This finding is in accord with the larger amounts of pigment in the first extracts. The first extracts contained, also, larger amounts of material, presumably protein, which may be flocculated by heating under conditions which do not inactivate the antigen.

Other extracts were prepared by treating fresh or dried parasites with N/10 barbiturate buffer (pH 8.5) at room or refrigerator temperature for varying time periods. Either method yielded active preparations containing 320 to 640 units per cc. Repeated extractions recovered additional antigen.

A highly active antigen was prepared by extracting the wet parasites obtained from one monkey first with 200 cc. of barbiturate buffer and then with 50 cc. The combined extracts were dialyzed against distilled water to remove salts, and subsequently concentrated in a semi-permeable membrane at 5°C. to 40 cc. Centrifugation yielded a supernate of 1280 units per cc.

Fresh parasites were treated with barbiturate buffer, pH 8.5, at room temperature for one hour. Ten per cent NaOH was added drop by drop, with gentle agitation until the parasites dissolved. A dark brown solution was obtained which was dialyzed against N/10 barbiturate buffer (pH 8.5) for 3 days at 5°C. Some pigment precipitated during dialysis. After centrifugation the supernate was tested for antigenic content and found to contain 800 units per cc.

Fresh parasites were dissolved in 0.096 N NaOH. The solutions were then adjusted to pH 7.0 with dilute HCl which precipitated most of the pigment and perhaps some other materials. The opalescent pale brown supernate was then concentrated in a semipermeable membrane at 5°C. to approximately 25 per cent of the original volume, a process requiring about 3 days. While antigenic, this preparation was not satisfactory because of its anticomplementary properties.

The authors enumerate various properties of the knowlesi antigens:

1. The antigenic material is of the nature of a protein-lipid complex. They have found that lipids extracted from wet or dried parasites are not antigenic; furthermore, barbiturate buffer extracts of lipid-free, dried parasites are inactive. Ether treatment of dried parasites, preliminary to saline extraction by freezing and thawing, reduces greatly or destroys the antigenic properties (Dulaney, Stratman-Thomas, and Warr, 1942). If wet parasites are first extracted with acetone containing 1 per cent HCl by volume, the residue does not yield antigen by any of the methods which are otherwise productive. No evidence of a carbohydrate in filtrates of hydrolysed parasite preparations has yet been demonstrated through use of copper reduction methods, but a carbohydrate factor has not been entirely eliminated.

2. The antigenic properties of saline, phosphate, or barbiturate buffer extracts are not destroyed by heating in a water bath at 56°C. for 30 minutes, 75°C. for 15 to 30 minutes, and 100°C. for 5 to 15 minutes. Heating at 56°C. for 30 minutes does not affect the antigen as shown by comparison with unheated samples, but exposure to the higher temperatures reduces the activity by two to three dilutions. Heating produces flocculation of pigmented

material to an extent roughly proportioned to the temperatures and times to which the preparation is exposed and subsequent centrifugation yields an opalescent supernate with amorphous brown deposit. They find that supernates from extracts heated at the higher temperatures are practically colorless. However, supernates from preparations containing large amounts of pigment are sometimes found to be inactive after heating at 75° to 100°C. which suggests that the antigen is adsorbed on the flocculated pigment and removed with centrifugation. They claim that neither heated nor unheated antigens are stable indefinitely.

3. The pigment may be materially reduced by freezing or by drying phosphate buffer extracts. Freezing will flocculate most of the pigment in phosphate buffer extracts with the exception of the first highly colored preparation. Drying of phosphate buffer extracts by means of a vacuum pump and rehydration by 0.9 per cent NaCl and centrifugation yield almost colorless antigens. They find these preparations to be almost as active, in some cases as active, as the original extracts. It now appears that dehydration of phosphate buffer extracts of wet or dried parasites may offer a highly efficient method of storing malaria antigen.

4. The malaria antigen does not dialyze when left in semipermeable membranes suspended in 0.9 per cent NaCl or water at 5°C.

Materials and Methods

Ducks

Adult ducks of the white Pekin variety were used in all the experiments. An average of 90 cc. of blood was obtained from each bird.

Malaria Parasites and Dosage

The malaria parasite is a strain designated 12A by the Committee on Terminology of Strains of Avian Malaria of the American Society of Parasitologists. It was supplied by Dr. R. I. Hewitt of this University.

Parasitized blood was drawn from the donor bird on the day of, or day preceding, the peak of parasitemia and diluted with an equal volume of 2% sodium citrate in physiological saline. In all the experiments the birds were inoculated with 8-10 cc. of blood containing 1-2 billion parasites per cc. The parasitized blood was always injected into the leg vein with a #23 gauge needle.

Parasite Counts

Blood smears were made on the fourth and fifth day subsequent to inoculation, and every day thereafter until the parasite count was 100% or greater. These smears were stained with a Wright-Giemsa combination stain. The number of parasites per 500 red blood cells was calculated. We

very rarely had occasion to bleed birds whose parasite count was below 100%.

Withdrawals

Blood was withdrawn directly from the heart with an #18 gauge needle and 20 cc. syringe.

Procedures for Complement Fixation Test

All complement fixation tests were performed by either one of 2 procedures.

A. The Kolmer-Wassermann technic consists of a 2-tube qualitative test in which 1/5 amounts of all reagents are used.* This is done in order to conserve antigen and to permit more tests with the same sample of serum. The serum is diluted 1:2½ by adding 0.45 cc. of saline to 0.3 cc. of serum. One-tenth cc. of the diluted serum is combined with 0.1 cc. of antigen and 0.2 cc. of complement representing 1/5 of the full 2 unit dose. The complement and hemolysin (amboceptor) have already been titrated before the complement fixation tests are set up. Controls of all reagents are included. After overnight incubation in the refrigerator and 10 minutes at 37°C., 0.1 cc. of a 2%

---

* This is the micro Kolmer test with minor modifications. (Kolmer, J. A., Technic of Kolmer Complement fixation tests for syphilis employing 1/5 amounts of reagents. Am. J. Clin. Path. 12:109-115, 1942)

suspension of sheep cells and 0.1 cc. hemolysin (carrying 1/5 of 2 units) are added, and readings made after further incubation for 30 minutes at 37°C. Kahn tubes are used for these tests. Readings of negative or strongly positive reactions are easy. Intermediate reactions may be checked by centrifugation and by comparison with standards.

B. The procedure recommended by the Army Medical School for use at Kennedy General Hospital was also employed for testing various sera. This included titration of complement and hemolysin as recommended by Maltner, Maltner and Wadsworth (Standard Methods, N. Y. State Laboratory), namely, the determination of the 50% hemolytic dose of complement by a colorimetric method and use of the optimal amboceptor unit. The test proper consisted of combining 0.1 cc. of serum, 0.2 cc. of antigen, 0.1 cc. saline, and 0.2 cc. of complement containing four 50% units. Antigen controls containing 2 doses of antigen and complement deterioration controls were included. After overnight incubation at refrigerator temperature, 0.4 cc. of sensitized sheep red blood cells were added and readings made after a second incubation period of 30 minutes at 37°C.

Method of Antigen Titration

The dose of antigen to use in complement fixation tests has to be determined with known positive and negative sera. The stock antigen is diluted in serial fashion and 0.1 cc. amounts combined with 0.1 cc. of serum (1:2½) and 0.2 cc. of complement that has already been titrated. After overnight incubation in the refrigerator and 10 minutes at 37°C. on the following morning, 0.1 cc. of a 2% sheep cell suspension and 0.1 cc. hemolysin (carrying 1/5 of 2 units) are added, and readings made after further incubation for 30 minutes at 37°C. The 0.1 cc. antigen of the highest dilution of antigen giving a 4-plus reaction with the positive serum is arbitrarily called the unit. Such a dilution must, of course, give no reaction with the negative serum or with the anticomplementary controls. The antigen dose for tests has consisted of 2-4 units.

Experimental

Ten experiments have been performed for the purpose of extracting P. lophurae parasite material which might serve as antigen in the complement fixation for malaria with human sera.

Experiment 1 -- Attempts to separate the malaria parasites from the nuclei of the red blood cells of ducks.

Our first experiments dealt with the problem of separating the parasites from the red blood cell nuclei on the basis of differences in specific gravity.

One hundred and sixty cc. of heavily parasitized blood was obtained from 2 adult ducks. After pooling, the blood was defibrinated by shaking it in a flask of glass beads. The cells were packed by centrifugation and the plasma removed by suction. The packed cells were washed two times with 0.85% saline and laked by treatment with 10 volumes of distilled water for 30 minutes at room temperature. Excess hemoglobin was removed by two washings with distilled water. We observed that the cells would clump together in a gel-like mass the more they were washed with distilled water. The laked cells were divided into 3 equal portions labeled A, B, and C.

A. Saturated sucrose solution was added to laked cells to give a series of dilutions ranging from 1:4 to 1:0.4. The trial volume was 10 cc. The control tube contained 10 cc. of laked cells. The cell-sucrose mixtures were stirred thoroughly and centrifuged at 3000 rpm for one hour. It was observed that there was no distinct layering of the sediment. However, we found that the

topmost portion was a shade darker than that nearer the bottom. Smears were made of both portions of the sediment and stained with a combination Wright-Giemsa stain. Studies of these smears revealed the nuclei-parasite ratio in the top portion to be approximately 1:2, whereas, that in the bottom portion was 1:1. It was found, however, that this difference in nuclei-parasite ratio of the 2 portions was not due to the separation of parasite from the cell stroma and nuclei, but was merely a result of indistinct layering of parasitized red blood cells over the non-parasitized ones. Only a comparatively few parasites were free from the web-like stroma surrounding the cell nuclei.

B. Laked parasitized cells were agitated with a mechanical stirrer after the same amounts of sucrose solution as given above had been added. Constant stirring for 1 hour followed by high speed centrifugation (3,000 rpm) for 1 hour failed to produce a distinct layering of the sediment. There was no increase in parasite-nuclei ratio by smear examination.

C. The procedure followed in (B) was repeated with the exception that the cell-sucrose mixtures were shaken vigorously by hand in flasks containing beads for 1 hour before high speed centrifugation was carried out.

This modification of the above procedure failed to improve the layering of sediment or parasite-nuclei ratio.

From these results, we are of the opinion that high speed centrifugation of laked parasitized cells in sucrose will not separate the parasites from the cell nuclei on the basis of differences in specific gravity. Clumping of the laked cells offers one explanation of the failure to obtain parasite-nuclei separation.

Experiment 2 -- Attempts to dissolve red blood cell nuclei with molar NaCl.

After realizing the difficulties involved in separating the parasites from the red blood cell nuclei, we considered the possibility of dissolving the cell nucleus with M NaCl and at the same time leaving the parasite intact. In our first approach to this problem, we undertook to dissolve the nuclear material of the normal, unparasitized duck red blood cells.

Twenty cc. of normal duck blood were collected in 5 cc. of citrated saline, centrifuged, and washed once with 0.9% saline. Approximately 5 cc. of packed cells were obtained which were laked in 100 cc. of 0.05% buffered saponin solution for 15 minutes at room temperature. The laked cells were centrifuged and washed twice in 0.85% saline. It was noted that the nuclear material swelled,

became viscous, and adhered together in a grey-white, gelatinous mass when washed with 0.85% saline. The cells were then poured slowly into 500 cc. of M NaCl while stirring with a mechanical stirrer. This mechanical agitation continued for 1 hour. At the end of that time the undissolved material which remained was centrifuged and treated for another hour in 300 cc. of M NaCl. After a third similar treatment practically all the nuclei had dissolved.

This procedure was repeated using parasitized duck red blood cells.

Three 8 weeks old white Pekin ducks with a parasitemia slightly more than 100% were exsanguinated. The blood was collected in 1/5 volume of citrated saline, centrifuged, and washed in 0.85% saline. Ten volumes of 0.05% saponin solution were added to the washed cells as the mixture was being agitated with a mechanical stirrer. At the end of 30 minutes the laked cells were centrifuged and the residue of nuclei-parasites was poured into 20-30 volumes of M NaCl while stirring with a mechanical stirrer. This agitation lasted 1 hour, and the undissolved material which remained after centrifugation was treated again. Smear examination of the residue after the first treatment with M NaCl showed neither formed parasites nor nuclei. There were only numerous strands and amorphous clumps of

lavender-and blue-staining material. Microscopic examination of the sediment after the second treatment with M NaCl showed essentially the same thing. The clumps were generally smaller in size. The sediment after the second treatment with M NaCl was grossly reduced. After the third treatment with NaCl the sediment was negligible in amount and microscopically revealed only small strands and clumps of the same lavender-and blue-staining material seen formerly.

We concluded that M NaCl not only dissolves the nucleus of the red blood cells, but also disintergrates, and possibly dissolves, the malaria parasite as well. This was borne out first by the fact that the smear examination of the sediment after the first treatment with M NaCl showed only amorphous clumps and strands of blue-staining nucleur material and lavender-staining cell stroma and parasite material. Secondly, the sediment was grossly reduced in amount after each treatment with M NaCl.

Experiment 3 -- Attempts to demonstrate an alcohol-ether soluble fraction of the P. lophurae parasite.

In this experiment we attempted to (1) prepare a lipid extract of the malaria parasite and (2) to determine if such treatment removed all activity from the residue.

Procedure

An adult duck exhibiting a 95% parasitemia was bled yielding 90 cc. of blood. This was defibrinated and the cells packed by centrifugation. After removing the plasma, we washed the packed cells two times with physiological saline. The washed cells were laked by treating with distilled water for 1 hour, and the parasites collected by centrifugation. After laking, the nuclei-parasite sediment was washed two times with physiological saline. We observed that the more the cells were washed with saline, the more viscous and gelatinous they became. In spite of the fact that each succeeding saline washing was definitely paler than the previous one, the red color of the nuclei-parasite mass remained about the same. Since we have noted that parasitized red blood cells when treated with either distilled water or physiological saline form a viscous gel-like mass, we were led to believe that the hemoglobin remaining behind after laking and washing red blood cells could either be within the red blood cell envelope or in the freed state but held within the gelatinous nuclei-parasite mass. Irrespective of the state in which the hemoglobin existed, it could not be completely removed from the nuclei-parasites. Ten volumes of freshly made alcohol-ether solution (3 parts 95% alcohol to 1 part ether) were added to the nuclei-parasite mass.

The mixture was shaken frequently for 1½ hours during refrigerator storage, and then allowed to remain in the icebox overnight.

On the following morning the material was centrifuged and the supernatant decanted into a petri plate which was left at room temperature until evaporation had occurred. It was then stored in the refrigerator until ready for use, at which time approximately 10 volumes of physiological saline were added to the dried, pale red residue. The parasite suspension was shaken frequently during 1 hour incubation at room temperature. The mixture was then centrifuged and the almost clear supernatant marked as antigen 1. The sediment which remained was insignificant in amount.

To the residue from which the lipid extract had been made was added 10 volumes of physiological saline. After thoroughly shaking and mixing, it was frozen and thawed six times with a dry ice-alcohol mixture. Centrifugation yielded an almost clear supernatant which was designated antigen 2.

These antigens were titrated with known positive and negative malarious sera diluted 1:2½. The P. knowlesi antigen served as a control.

TABLE I

SHOWING THE RELATIVE ACTIVITY OF EXTRACTS OF P. LOPHURAE PARASITES

Antigens	Sera Malaria +	Sera Malaria −	No Serum
Lophurae #1 Alcohol-ether extract (1:2)	1+	−	−
Lophurae #2 Saline extract of residue (1:2)	−	−	−
Knowlesi Saline extract (1:2)	4+	−	−

Antigen 1 proved faintly active, while antigen 2 failed to fix complement with known positive malarious serum. The *P. knowlesi* antigen gave a 4-plus reaction with the same positive serum. Neither of the *P. lophurae* preparations gave positive reactions with either the antigen or the serum controls.

We conclude that alcohol-ether treatment of wet parasites, preliminary to saline extraction by freezing and thawing, reduces greatly or destroys the antigenic properties of the extract. Furthermore, the alcohol-ether extracts are not satisfactory antigens.

Experiment 4 -- The use of buffered saponin and its value as a hemolytic agent in the preparation of *P. lophurae* antigens.

The hemolytic properties of saponin are well known. We therefore decided to use this reagent in an attempt to completely lake parasitized red blood cells of ducks since it had been found that water alone did not produce complete hemolysis.

Procedure

Two heavily parasitized adult ducks were bled by heart puncture. The blood was defibrinated by shaking in a flask containing glass beads. The cells were packed by centrifugation and washed two times with physiological

saline. The washed red blood cells were divided into 2 portions designated 1 and 2.

The cells labeled 1 were laked by treatment with 0.05% buffered saponin solution (0.05% gram powdered saponin per 100 cc. buffered phosphate of pH 7.3) for 45 minutes, after which they were centrifuged and washed four times with physiological saline. The same viscous gel-like mass resulted as previously described.

The cells labeled 2 were laked by treating with distilled water for 45 minutes. The preparation was then centrifuged and the gel-like mass washed as described for lot 1.

When the laked cell washings of 1 and 2 were compared as to color intensity, it was evident that the washings of the saponin-laked cells were darker red than the washings of the cells laked with distilled water. Furthermore, the laked cell mass designated 1 was a shade paler than that labeled 2. This observation indicates that the 0.05% buffered saponin solution lakes red blood cells more thoroughly than distilled water.

Smear examination of both laked samples showed the parasites in each to be intact despite alterations in morphology. The pigment was not dispersed, but arranged in the same pattern as in untreated parasites.

TABLE II

COMPARISON OF THE ACTIVITY OF SALINE EXTRACTS OF PARASITIZED CELLS LAKED WITH SAPONIN AND WITH WATER

Antigen (1:2)		Sera (1:2½)		
Number	Method of Preparation	+ Malaria	− Malaria	No Serum
Lophurae #1	Saline extracts of parasitized cells laked in 0.05% buffered saponin	−	−	−
Lophurae #2	Saline extracts of parasitized cells laked in distilled water	2+	−	−
Knowlesi	Saline extracts	4+	−	−

Since both samples were quite gelatinous in consistency, they were divided numerous times with applicator sticks and frozen and thawed six times in 10 volumes of physiological saline. We then placed the 2 samples in the icebox overnight. On the following morning they were centrifuged and the dark red supernates labeled antigens 1 and 2.

Titrations were carried out on these 2 antigens with the P. knowlesi antigen control. Known positive and negative malarious sera were used.

Table II shows antigen 2 to have slight antigenic activity, while antigen 1 is inactive. Neither preparation was anticomplementary. The P. knowlesi antigen gave a 4-plus reaction in a 1:2 dilution which represented the dilution routinely employed for test.

We conclude that 0.05% saponin hemolyzed red blood cells more completely than distilled water; however, the concentration of saponin necessary for red blood cell hemolysis modifies the antigenic activity. This finding is substantiated by similar experiments on the P. knowlesi antigen in this laboratory.

Experiment 5 -- The use of barbiturate buffer as an extracting reagent with and without water bath incubation of parasitized red blood cells.

In this experiment we used barbiturate buffer as an extracting reagent. Since it was believed that water-bath incubation might increase the fragility of the red blood cells, we extracted parasites with and without coincident water-bath incubation.

Procedure

Three adult ducks with a 100% parasitemia were exsanguinated. The blood was citrated and pooled. The cells were packed by centrifugation at 2,000 rpm for 30 minutes, and the plasma removed. The packed cells were divided equally as to volume into 4 parts, from which 5 different extracts were to be made, and then each part was washed two times with 20 volumes of physiological saline. The 4 lots of cells were treated as follows:

1. The washed cells were laked by addition of 20 volumes of distilled water. After incubation at room temperature for 45 minutes, they were centrifuged and washed two times with 10 volumes of physiological saline in an attempt to remove any excess hemoglobin from the laked cells. Ten volumes of barbiturate buffer were then added to the cells, after which they were stored in the

refrigerator for 48 hours with frequent shaking during the time. After 48 hours, the mixture was centrifuged and a dark red supernatant was separated which was labeled B-W. This preparation served as a control for other barbiturate buffer preparations made from parasites subjected to water-bath incubation.

2. Lot II of the washed, packed cells was incubated overnight at 37°C. in the water-bath, after which they were laked by treatment with 20 volumes of distilled water for 45 minutes. The mixture was centrifuged and the deep red supernate removed. Ten volumes of physiological saline were added to the laked cells. After mixing thoroughly, the suspension was centrifuged and the red supernatant decanted. This saline washing was repeated again in an attempt to wash the cells free of hemoglobin. To the sediment of laked cells was added 10 volumes of barbiturate buffer, after which they remained in the refrigerator with frequent shaking for 48 hours. At the end of this period the mixture was centrifuged, and a dark red supernate was obtained and labeled B-W-1.

3. The washed cells (Lot 3) were incubated overnight at 37°C. in 10 volumes of distilled water. On the following morning the cells were packed by centrifugation and the deep red supernatant removed. This supernatant appeared darker than any yet seen as a result of laking with distilled water.

The laked cells were washed two times with physiological saline as described above. The washings appeared a shade paler than those of the 2 preceding portions. Ten volumes of barbiturate buffer were added to the laked cells. Likewise, this remained in the icebox for 48 hours, during which time it was shaked frequently. The mixture was then centrifuged and the red supernatant was labeled B-W-2 to be used as antigen.

4. The washed cells were laked by treatment with 20 volumes of distilled water for 45 minutes, after which the excess hemoglobin was removed by two washings with 10 volumes of physiological saline. The laked cells were then incubated in the water-bath in 10 volumes of barbiturate buffer. On the following morning, the mixture was centrifuged and the dark red supernatant obtained was designated B-W-3.

To the residue obtained was added an additional 10 volumes of barbiturate buffer, and this mixture was also placed in the icebox where it was shaken at intervals during a 48 hour period. At the end of this time it was centrifuged and the red supernate marked B-W-4.

Smear examination of each of the cell samples following overnight incubation showed the parasites to be intact and there appeared to be little change in morphology.

We observed a greater number of laked cells in the samples that had been subjected to water-bath incubation than in the unincubated control.

These preparations were tested with known positive and negative malarious sera diluted 1:2½. The antigens were used in dilutions of 0 to 1:32. The 2 highest dilutions of antigen giving any type of reaction are shown in Table III.

Antigens 1 and 2 gave positive reactions in the lower dilutions of positive malarious sera. These reactions, however, are not significant since the antigen controls were anticomplementary in the same dilutions. The other antigens were either inactive or only so slightly active as to be insignificant.

We conclude from these results that:

1. Barbiturate extracts of wet parasites, though apparently more antigenic than any tested previously, are, however, anticomplementary and give false-positive reactions with pooled negative sera.
2. Water-bath incubation increases the fragility of red blood cells.
3. Water-bath incubation modifies the antigenic properties of the malaria parasite.

TABLE III

ACTIVITY OF BARBITURATE BUFFER EXTRACTS OF PARASITES TREATED WITH AND WITHOUT INCUBATION

Antigen		Two Highest Dilutions of Antigen giving + Reactions			
Name	Method of Preparation	Antigen Control		+Malaria Serum		−Malaria Serum	
Lophurae #1	Barbiturate buffer extract of wet parasites	1:4	1+	1:4	2+	0	3+
		1:8	±	1:8	1+	1:2	−
Lophurae #2	Barbiturate buffer extract of wet unlaked parasitized cells after overnight incubation	1:2	3+	1:4	3+	1:2	3+
		1:4	2+	1:8	1+	1:4	±
Lophurae #3	Barbiturate buffer extracts of wet parasites after overnight incubation in distilled water	−		−		−	
Lophurae #4	Barbiturate buffer extract of wet parasites after overnight incubation in barbiturate buffer	−		−		−	
Lophurae #5	Barbiturate buffer extract of residue of #4	−		0	2+		−
				1:2	1+		
Knowlesi	Saline extract	−		1:8	3+		−
				1:16	±		

Experiment 6 -- Barbiturate buffer and phosphate buffer compared as extracting reagents of the wet P. lophurae parasites.

In this experiment phosphate buffer was used as extracting reagent and its efficiency compared to that of barbiturate buffer.

Procedure

The bloods of 2 heavily parasitized (135%) adult ducks was pooled in 1/5 volume of 2% citrated physiological saline. The cells were packed and the plasma removed. The cells were washed two times with 0.85% saline. Twenty volumes of distilled water were added to the washed cells and the mixture allowed to stand for 45 minutes at room temperature during the laking process. After washing two times with 0.85% saline the laked cells were divided into 2 equal portions designated A and B.

A. The laked cells were treated with 10 volumes of barbiturate buffer for 1 hour at room temperature during which time they were frequently shaken.

B. To the other portion of laked cells was added 10 volumes of phosphate buffer, which stood at room temperature for 1 hour with frequent shaking.

The 2 samples were centrifuged and the dark red supernates labeled B-W and P-W, respectively.

TABLE IV

COMPARISON OF BARBITURATE BUFFER EXTRACTS AND PHOSPHATE BUFFER EXTRACTS OF WET PARASITES

Name	Antigen Method of Preparation	Two Highest Dilutions of Antigen giving + Reactions: Antigen Control		+ Malaria Serum		− Malaria Serum	
B-W	Barbiturate buffer extracts of wet *P. lophurae* parasite	1:4	1+	1:4	2+	0	3+
		1:8	±	1:8	1+	1:2	—
P. W.	Phosphate buffer extracts of wet *P. lophurae* parasite	1:4	1+	1:4	2+	0	2+
		1:8	±	1:8	1+	1:2	-
Knowlesi	Saline extract		-	1:2	3+		—
				1:16	2+		

When these antigens were titrated with known positive and negative malarious sera, the two fractions were found to have equal activity. Both were anti-complementary in low dilutions.

From the results obtained we are of the opinion that:

1. Either barbiturate buffer or phosphate buffer serves equally well as an extracting agent of wet P. lophurae parasites.
2. The wet P. lophurae parasite is unsatisfactory material from which to make antigenic extracts.

Experiment 7 -- The use of saline as an extracting agent for P. lophurae parasites (dried).

It has been shown by previous experiments that neither saline, barbiturate buffer, nor phosphate buffer extracts of wet P. lophurae parasites make satisfactory antigens. Since it has been found that extracts of dried P. knowlesi parasites are more antigenically active than the same extracts of the wet parasite, we had reason to believe the same would hold true in case of P. lophurae. With this possibility in mind, it was decided to make saline extracts of dried P. lophurae parasites.

The following types of saline extracts of dried parasites were prepared:

1. Saline extracts of unlaked dried parasitized cells made by freezing and thawing.
2. Saline extracts of unlaked dried parasitized cells made at icebox temperature.
3. Saline extracts of laked dried parasitized cells made by freezing and thawing.
4. Saline extracts of laked dried parasitized cells made at icebox temperature.

Procedure

Two adult ducks with a parasitemia of 120% were bled directly from the heart. The citrated bloods were pooled and centrifuged for 30 minutes at 2000 rpm. The plasma was removed and the packed cells were divided into 2 equal lots labeled A and B.

The unwashed cells of lot A were poured into a petri plate, spread into a thin layer, and dried *in vacuo* for 8 hours.

The packed cells (Lot B) were washed two times with 10 volumes of physiological saline as usual. They were then laked with 20 volumes of distilled water. At the end of 1 hour the laked cells were freed of excess hemoglobin by 2 washings with 0.85% saline. The cells, viscous as usual, were poured into a petri plate, spread into a thin layer, and dried *in vacuo* along with lot A.

Smear examinations of the parasites taken from either lot after the drying and rehydrating process showed them to closely resemble those made of parasites prior to drying.

The dried cells of both samples were ground to a fine powder in an agate mortar and placed in a dessicator until ready for use. Lot A was of a darker red color than lot B. From each lot were taken two 0.5 gram portions marked A 1, A 2, B 1, and B 2.

A 1. -- Ten cc. of 0.85% saline were slowly added to 0.5 gram of dried unlaked cells while the parasite material was ground in an agate mortar. The material was frozen and thawed six times and then centrifuged at high speed. The dark red supernate was decanted and labeled UL-D-FT-S-1.

To the sediment was added an additional 10 cc. of 0.85% saline. This was thoroughly mixed and placed in the icebox for 48 hours. Centrifugation revealed a red supernate which was labeled UL-D-FT-S-2.

A 2. -- The second portion of dried unlaked cells was processed in a similar way as the first except the extracts were made at icebox temperature for a period of 30 minutes, centrifuged, and the dark red supernate used as antigen UL-D-IT-S-1.

The sediment was extracted for 48 hours with 10 cc. 0.85% saline at refrigerator temperature. The red supernate was labeled UL-D-IT-S-2.

B 1 and B 2 -- The same procedure carried out for portions A 1 and A 2 was repeated with the 2 0.5 gram portions of dried cells that had been previously laked. Four additional extracts were obtained; viz., L-D-FT-S-1, L-D-FT-S-2, L-D-IT-S-1, and L-D-IT-S-2. All of these extracts were definitely less red than those prepared from dried unlaked cells.

These antigen titrations are of great interest in that they show none of the extracts to be anticomplementary. It is also apparent from Table V that much antigenic material is left in the residue following the first extraction with saline. One will note that the antigenic activity has been increased throughout the entire series of antigens tested in this experiment. The drying process can be the only explanation for this general increase in sensitivity.

From the results in Table V we conclude that:

1. Saline extracts made from dried parasites are definitely more antigenic than are those made from wet parasites.
2. Drying either the laked or the unlaked parasitized cell apparently modifies the nuclei-parasite material in such a way so as to eliminate the anticomplementary fraction.
3. Laking cells prior to drying is recommended not only for the purpose of eliminating much of the

TABLE V

SHOWING THE ACTIVITY OF SALINE EXTRACTS OF DRIED P. LOPHURAE PARASITES

Antigen		Two Highest Dilutions of Antigen giving + Reactions		
Name	Method of Preparation	Antigen Control	+ Malaria Serum	− Malaria Serum
UL-D-FT-S-1	First saline extract of dried unlaked cells after freezing and thawing	−	1:4 3+ 1:8 1+	−
UL-D-FT-S-2	Second Saline extract	−	1:4 4+ 1:8 1+	−
UL-D-IT-S-1	First saline extract of dried unlaked cells at icebox temperature	−	1:4 3+ 1:8 2+	−
UL-D-IT-S-2	Second saline extract	−	1:4 2+ 1:8 1+	−
L-D-FT-S-1	First saline extract of dried laked cells after freezing and thawing	−	1:8 3+ 1:16 2+	−

TABLE V (Cont'd)

SHOWING THE ACTIVITY OF SALINE EXTRACTS
OF DRIED P. LOPHURAE PARASITES

Antigen		Two Highest Dilutions of Antigen giving + Reactions		
Name	Method of Preparation	Antigen Control	+Malaria Serum	−Malaria Serum
L-D-FT-S-2	Second Saline extract	−	1:4 4+ 1:8 2+	−
L-D-IT-S-1	First saline extract of dried laked cells at icebox temperature	−	1:8 4+ 1:16 1+	−
L-D-IT-S-2	Second saline extract	−	1:4 3+ 1:8 2+	−
Knowlesi	Saline extract	−	1:16 4+ 1:32 3+	−

hemoglobin from the extract, but also because the sensitivity is increased.

4. Freezing and thawing releases at times only slightly more antigenically active material and at other times less than that obtained by simple extraction.

5. Freezing and thawing, though a recommended procedure in the preparation of the *P. knowlesi* antigen, is not a practical and worthwhile procedure in the preparation of *P. lophurae* extracts. It is our opinion that the viscous nuclear substance serves as insulating material for the parasite in such a way that the latter exhibits an even more remarkable resistance to disruption by the freezing and thawing process than does the freed *P. knowlesi* parasite.

Experiment 8 -- The effect of $NaHCO_3$-HCl treatment of dried *P. lophurae* parasites.

It has been shown in the preceeding experiments and by other workers (3) that either a barbiturate buffer or a phosphate buffer extract is more active than a saline extract of malaria parasite material. We have reasons to believe that this fact can be explained merely on the basis of a difference in pH of the extracting reagents.

With this possibility in mind, we attempted to make an extract of the dried P. lophurae with an alkaline solution of M $NaHCO_3$ and to subsequently neutralize the mixture with N HCl.

Procedure

One heavily parasitised (110%) adult duck was exsanguinated. To the blood obtained was added 1/5 volume of 2% citrated saline. The citrated blood was centrifuged, the plasma removed, and the packed cells washed two times with 10 volumes of 0.85% saline. The washed cells were then laked by subjection to 20 volumes of distilled water for 1 hour. The laked cells were freed of excess hemoglobin as usual by washing two times with 10 volumes 0.85% saline. The nuclei-parasite mass was dried in vacuo as previously described. The dried parasite material was worked into a fine powder with an agate mortar. Approximately 5 volumes of M $NaHCO_3$ were added slowly to the ground material and mixed thoroughly with a mortar and pestle. A gel-like mass resulted. N HCl was then added with care to the alkaline suspension until the mixture was approximately neutral. We found difficulty in obtaining satisfactory neutrality of the mixture because of the viscous nuclei-parasite mass. After remaining in the refrigerator overnight, the mixture was centrifuged and the red supernatant used as antigen.

TABLE VI

SHOWING THE ANTIGENIC ACTIVITY OF A $NaHCO_3$-HCl EXTRACT OF DRIED *P. LOPHURAE* PARASITES

Antigen		Two Highest Dilutions of Antigen giving + Reactions		
Name	Method of Preparation	Antigen Control	+ Malaria Serum	- Malaria Serum
$NaHCO_3$-HCl	$NaHCO_3$-HCl extract of dried *P. lophurae* parasites	-	1:4 4+ 1:8 3+	-
Knowlesi	Saline extract	-	1:8 4+ 1:16 3+	-

Titration of this antigen as shown in Table VI reveals that this extract was antigenically active. It was not anticomplementary.

We conclude that even though extracts obtained by $NaHCO_3$-HCl treatment of dried parasites show sensitivity, the technicalities of the neutralizing process make this procedure less practical than others cited previously.

Experiment 9 -- Barbiturate buffer and phosphate buffer as extracting agents for dried P. lophurae parasites.

We are of the opinion that the drying process renders the nuclei-parasite material more antigenic. We were yet enthusiastic to know how much of the antigenically active substance bound in the dried parasite could be released by extracting with barbiturate buffer and phosphate buffer. Both laked and unlaked parasitised cells were dried and treated with these extracting agents.

Procedure

The bloods of 2 heavily parasitised (120%) ducks were citrated and pooled as previously done. The plasma and cells were separated by centrifugation and the plasma removed. The packed cells were divided into 2 equal volumes.

The first portion of packed cells was washed two times with 10 volumes of physiological saline, after which they were poured into a petri plate and dried in vacuo.

The second portion of packed cells was likewise washed two times with 10 volumes of physiological saline. The cells were then laked by treating with 20 volumes of distilled water for 1 hour. At the end of this time, the laked cells were centrifuged and the packed nuclei-parasite mass washed free of excess hemoglobin with the usual physiological saline treatment. This was repeated once. The nuclei-parasite material was then poured into a petri plate and dried in vacuo in conjunction with the unlaked cells of lot 1.

The dried parasite material of both lots was treated as formerly by grinding thoroughly in an agate mortar to a fine powder. Four separate 0.5 gram portions were taken from each lot and labeled Ia, Ib, Ic, Id and IIa, IIb, IIc, IId. The four samples were treated as follows:

I a. To 0.5 gram of dried unlaked parasitized cells were added 10 volumes of barbiturate buffer. This mixture was kept in the refrigerator for 24 hours. At the end of this time the material was centrifuged and the red supernatant was decanted and labeled B-D-UL-24.

I b. To 0.5 gram of the dried unlaked parasitized cells were added 10 volumes of barbiturate buffer. Instead of centrifuging at the end of 24 hours, we separated the barbiturate buffer extract from the residue at the end of

1 hour at room temperature by high speed centrifugation for 30 minutes. The supernatant was labeled B-D-UL-1.

I c. To another 0.5 gram sample of the unlaked portion of dried parasitized cells was added 10 volumes of phosphate buffer. This mixture, which remained in refrigerator storage for 24 hours, was then centrifuged at high speed for 30 minutes and the supernate separated and saved as P-D-UL-24.

I d. To the remaining 0.5 gram of dried unlaked parasitized cells were added 10 volumes of phosphate buffer. As in I b, this mixture, after remaining at room temperature for 1 hour, was centrifuged. The supernate obtained was marked P-D-UL-1.

II a, IIb, II c, II d. The above procedure as described in I a, I b, I c, and I d was carried out on the four 0.5 gram samples of dried, laked, parasitized cells. Four additional extracts were obtained bearing the labels: B-D-L-24, B-D-L-1, P-D-L-24, and P-D-L-1.

Titrations were carried out on these 8 extracts with known positive and negative malarious sera. From Table VII we see that the activity of barbiturate buffer and phosphate buffer extracts of dried parasitized cells is about equal. It is apparent that dried laked cells make better parasite material than dried unlaked cells. The

TABLE VII

COMPARISON OF BARBITURATE BUFFER EXTRACTS
AND PHOSPHATE BUFFER EXTRACTS OF DRIED PARASITES

Antigen		Two Highest Dilutions giving + Reactions			
Name	Method of Preparation	Antigen Control	+Malaria Serum		−Malaria Serum
B-D-UL-24	24 Hour barbiturate buffer extract of dried unlaked parasitized cells	−	1:16	3+	−
			1:32	1+	
B-D-UL-1	1 hour barbiturate buffer extract of dried unlaked parasitized cells	−	1:16	3+	−
			1:32	2+	
P-D-UL-24	24 hour phosphate buffer extract of dried unlaked parasitized cells	−	1:16	3+	−
			1:32	1+	
P-D-UL-1	1 hour phosphate buffer extract of dried unlaked parasitized cells	−	1:16	4+	−
			1:32	3+	
B-D-L-24	24 hour barbiturate buffer extract of dried laked parasitized cells	−	1:32	4+	−
			1:64	1+	
B-D-L-1	1 hour barbiturate buffer extract of dried laked parasitized cells	−	1:32	4+	−
			1:64	3+	

TABLE VII (Cont'd)

COMPARISON OF BARBITURATE BUFFER EXTRACTS
AND PHOSPHATE BUFFER EXTRACTS OF DRIED PARASITES

Antigen		Two Highest Dilutions giving + Reactions		
Name	Method of Preparation	Antigen Control	+Malaria Serum	−Malaria Serum
P-D-L-24	24 hour phosphate buffer extract of dried laked parasitized cells	−	1:32 4+ 1:64 1+	−
P-D-L-1	1 hour phosphate buffer extract of dried laked parasitized cells	−	1:32 4+ 1:64 3+	−
Knowlesi	Saline extract	−	1:16 4+ 1:32 3+	−

one-hour extracts at room temperature are just as potent and in some cases even more active than 24 hour extracts at refrigerator temperature. None of the antigens are anticomplementary.

From these results we conclude that:

1. Barbiturate buffer extracts and phosphate buffer extracts of dried parasitized cells are of the same antigenic activity.
2. Barbiturate buffer extracts and phosphate buffer extracts of dried parasitized cells are definitely more antigenic than saline extracts of the same.
3. Dried laked cells are better parasite material than dried unlaked cells.
4. One-hour extracts at room temperature make just as effective, or at times more effective, antigens than 24 hour extracts at refrigerator temperature.

Experiment 10 -- The effect of citrate-dextrose treatment of parasitized duck red blood cells preliminary to drying and extracting with phosphate buffer.

We have found it impossible to completely lake the red blood cells of ducks through use of reagents employed. A series of papers have appeared regarding the preservation,

storage, and hemolysis of human red blood cells. Outstanding among the workers are Maizels (17), Mollison (24), and Whittaker (20). From extensive investigation on the storage of human red blood cells, then have found certain preservatives to be less effective than others. As a matter of fact, they found various reagents such as certain concentrations of citrate-dextrose solutions to promote, rather than prevent, hemolysis. This observation suggested that we expose parasitized duck red blood cells with such reagents prior to laking with distilled water. We hoped by such treatment that the red blood cells would hemolyse more completely.

Procedure

Ninety cubic centimeters of heavily parasitized blood (120%) was obtained from one adult duck, and was citrated with 1/5 volume of 2% sodium citrate in physiological saline. The cells were packed by centrifugation and the plasma removed. The packed cells were stirred thoroughly, and two 10 cc. portions were removed.

To the first 10 cc. portion was added 10 cc. of 2.5% sodium citrate, since it has been found that this strength of citrate is isoplethecontic with blood. The cell-citrate mixture remained for 4 hours at refrigerator temperature during which time it was frequently stirred.

The mixture was then centrifuged and the citrate removed. An additional 10 cc. of sodium citrate, containing 1 gram of dextrose, was stirred with the packed cells and the mixture allowed to remain in the refrigerator overnight. On the following morning, the citrate-dextrose-treated cells were packed by centrifugation and the citrate-dextrose removed. To the cells were added 6-8 volumes of distilled water. After shaking frequently for 30 minutes at room temperature, we centrifuged the laked cells at high speed for 30 minutes. The deep red supernatant was removed. For the first time we observed that the laked cells were not viscous and gel-like in nature. This quality of the cells afforded a more thorough subsequent washing with saline just as it had a more complete hemolysis with distilled water. The washed nuclei-parasite sediment was a dark brown color and thick, but not viscous and adherent.

The second 10 cc. portion was similarly treated except that a 5% sodium citrate solution was used instead of a 2.5%. Likewise, 5% dextrose was added as in the first 10 cc. portion because it has been found that large amounts (5-7%) of dextrose increase hemolysis. The washed nuclei-parasite sediment in this case differed in no respect from the first sediment as to color and consistency.

Smear examination of both nuclei-parasite sediments showed relatively fewer cells that had escaped hemolysis.

These cells showed an increase in size over smears on normal unlaked cells which had never been exposed to citrate-dextrose and distilled water. There was no essential difference in the smears of the 2 sediments.

Both nuclei-parasite sediments were dried *in vacuo* in the usual way. The dried parasite material was worked into a fine powder by grinding it in an agate mortar.

Two 0.5 gram samples were taken from either portion and extracted as follows:

To the 0.5 gram sample of portion 1 was slowly added 10 cc. of PO_4 buffer. The 2 were mixed thoroughly by grinding in a mortar for 10 minutes, after which the brownish-black mixture remained at refrigerator temperature for 6 hours. It was then centrifuged at 3000 rpm for 30 minutes. The very pale red, opalescent supernatant was decanted and labeled P-D-L-CD-1.

To the 0.5 gram sample of portion 2 was added the same volume of PO_4 buffer. The procedure for obtaining the final extract was identical to that employed for the above extract. Another very pale red, opalescent supernatant was obtained which was marked P-D-L-CD-2.

An antigen was prepared from normal unparasitized duck cells by using the same procedure as that carried out above. A 2.5% sodium citrate solution was used. We

TABLE VIII

ACTIVITY OF PHOSPHATE BUFFER EXTRACTS OF CITRATE-DEXTROSE TREATED PARASITIZED CELLS

Antigen		Two Highest Dilutions Giving + Reactions					
Name	Method of Preparation	Antigen Control		+ Malaria Serum		−Malaria Serum	
P-D-L-CD-1	Phosphate buffer extract of dried laked parasitized cells treated previously by citrate (2.5%)-dextrose (5%)	0	4+	1:64	4+	0	4+
		1:2	−	1:128	2+	1:2	−
P.D-L-CD-2	Phosphate buffer extract of dried laked parasitized cells treated previously by citrate (5%)-dextrose (5%)	0	4+	1:64	4+	0	4+
		1:2	−	1:128	1+	1:2	−
N-D-A	Phosphate buffer extract of dried laked normal cells treated previously by citrate (2.5%)-dextrose (5%)		−		−		−
Knowlesi	Saline extract		−	1:16	4+		−
				1:32	3+		

employed the use of a normal duck cell antigen in this experiment for the purpose of determining whether the red blood cells *per se* of the duck is antigenic.

Titrations of the 2 parasite extracts with known positive and negative malarious sera together with the extract made from the red blood cells of normal ducks are shown in Table VIII.

By the results shown, it is evident that there is no appreciable difference in the activity of the 2 phosphate buffer extracts. It is apparent that either is more active than any previously obtained. Both extracts were anticomplementary only in the 0 dilution. The normal duck cell *per se* is apparently not antigenically active. One of the most striking facts about this experiment that is not revealed in the table is the preparation of an active extract whose color is sufficiently pale so that readings of the titrations can be made with greater accuracy.

We conclude from these results that:

1. The treatment of parasitized cells with citrate-dextrose is worthwhile and practical since we believe such treatment increases red blood cell hemolysis.
2. Normal duck cells *per se* are apparently antigenically inactive.

We hesitate to state that this statement is the

final word, but we would rather recommend that additional studies be made regarding the antigenic properties of the red blood cells of normal ducks.

Preliminary tests for complement fixation using human sera are now being carried out in our laboratory, but the number of tests have not been sufficient to draw any specific conclusions at this time. With the *P. lophurae* antigen as prepared in this experiment, many of these tests have shown *P. lophurae* to be more active than *P. knowlesi*. The results of others are just the reverse.

Summary and Conclusions

In our first approach to this problem we attempted to separate the malaria parasite from the red blood cell nuclei. This was done because we had found the *P. knowlesi* antigen to be highly successful when extracted from the parasites from laked monkey cells. All attempts failed, and we were forced to look for some other method.

It seemed reasonable to try to dissolve the red blood cell nuclei. Molar NaCl was used in several attempts to dissolve the nuclei of both the red blood cells of normal and parasitised ducks. We were able to almost completely dissolve the nuclei of red blood cells of normal ducks, but when we attempted to subject the nuclei-parasite mixture

to the same treatment, we found the parasite to be either disintegrated or dissolved.

Having failed in all attempts to either separate or dissolve the nuclei from the parasites, we set out to make various extracts of the P. lophurae parasite in the presence of the nuclei of the red blood cell.

First, an attempt was made to prepare a lipid extract by alcohol-ether treatment of wet parasite material. We made saline extracts from the residue from the alcohol-ether extraction of the parasite mass. We found that lipids extracted from the wet parasites are not antigenic; furthermore, saline extracts of the lipid-free residue are also inactive.

Heretofore we had been unable to completely lake the red blood cells of ducks with distilled water, so we resorted to the use of buffered saponin in an attempt to overcome this difficulty. We found that 0.05% buffered saponin hemolyses red blood cells more completely than distilled water; however, the concentration of saponin necessary for red blood cell hemolysis modifies the antigenic activity of the parasite. Saline extracts of parasitized cells laked by both methods (distilled water; saponin) were slightly antigenic and inactive, respectively. They were not anticomplementary.

In a further attempt to promote complete hemolysis of the red blood cells we used water-bath incubation of parasitized red blood cells. It was believed that water-bath incubation increases the fragility of the red blood cells, and hence, makes it more amenable to hemolysis. We found this to be the case; however, such treatment modifies the antigenic properties of the parasite.

We then tried barbiturate buffer and phosphate buffer extracts of wet parasitized cells. The positive reactions which were obtained with either barbiturate buffer or phosphate buffer extracts of wet parasitized cells are insignificant since similar reactions occurred with the antigen controls. All attempts of making various extracts from wet parasitized red blood cells had been unsuccessful.

Since we had found that extracts made from dried P. knowlesi parasites are far more active than those obtained from wet parasites, we had reason to believe the same might be true in the case of P. lophurae. We next decided to prepare extracts of dried P. lophurae parasite material with saline, barbiturate buffer, and phosphate buffer. Both laked and unlaked parasitized cells were subjected to these extracting reagents. We found relatively more activity in the barbiturate buffer and phosphate buffer extracts than in the saline antigens. The drying process apparently

modifies the nuclei-parasite material in such a way that the anticomplementary fraction is eliminated, since none of these antigens were anticomplementary.

We found that only a portion of the active parasite material is removed with one extraction. The freezing and thawing process was tried, but was dismissed as a routine procedure.

We next attempted to make an extract of dried P. lophurae parasites with M $NaHCO_3$ and to subsequently neutralize the mixture with N HCl. Although this antigen showed activity, we are not in favor of this procedure because of technical difficulties involved in the neutralizing process.

Aided by the reports of various English workers on the preservation and storage of blood, we were lead to believe that treating wet parasitized cells in citrate-dextrose solution prior to laking would more completely hemolyze the red blood cells. This was done, and we observed for the first time that the laked and washed parasite sediment was no longer gel-like and viscous. We believe that the increased hemolysis which was evident was due in great part to the disappearance of the gel-like consistency of the parasite mass. Not only was the antigen

obtained more desirable because of its pale brown color, but also because it was antigenically more active than any yet obtained.

In conclusion, we are of the opinion that:

1. Parasites cannot be separated from the nuclei of red blood cells by high speed centrifugation upon the basis of differences in specific gravity.
2. The nuclei of parasitized red blood cells cannot be dissolved irrespective of the parasite with M NaCl.
3. Lipid extracts of wet parasites are inactive.
4. Saline extracts of lipid-free residues are inactive.
5. Saponin modifies the antigenic properties of the malaria parasite.
6. Water-bath incubation, though increasing the fragility of the red blood cell, modifies the antigenic properties of the parasite.
7. Dried P. lophurae parasites are preferred over wet P. lophurae parasites for making antigenic extracts.
8. Dried laked parasitized cells are preferred over dried unlaked parasitized cells not only because the former is more antigenically active, but also

because it produces a paler red-colored antigen.

9. Barbiturate buffer or phosphate buffer extracts are more active than saline extracts.

10. $NaHCO_3$-HCl extracts of dried P. lophurae parasites possess antigenic activity but are not to be recommended for routine use because of technical difficulties in the process of preparation.

11. Wet parasitized cells are more completely laked when they are treated with citrate-dextrose. Such treatment does not alter the antigenic properties of the malaria parasite.

12. Phosphate buffer extracts of the red blood cells of normal ducks are inactive.

Present indications are that the P. lophurae antigen may fulfill the same function as the P. knowlesi antigen. If this proves to be the case, we shall have a readily available and relatively cheap source of antigen for complement fixation.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Coggeshall, L. T., The complement fixation reaction as a diagnostic aid in malaria, Proc. Indiana Acad. Sci. 50:53-55, 1941. Abstracted in Trop. Dis. Bull. 39:118-119, 1941.

2. Coggeshall, L. T. and Eaton, M. D. The complement fixation reaction in monkey m laria, J. Exper. Med. 67:871-882, 1938.

3. Dulaney, A. D. and Morrison, D. B. On the preparation and properties of antigens from Plasmodium knowlesi, Amer. J. Trop. Med. 24:323-326, 1944.

4. Dulaney, A. D. and Stratman-Thomas, W. K. Complement fixation in human malaria. I. Results obtained from various antigens, J. Immunol. 39:247-255, 1940.

5. Dulaney, A. D., Stratman-Thomas, W. K., and Warr, O. S. The diagnostic value of complement fixation in malaria, J. Infec. Dis. 70:221-225, 1942.

6. Eaton, M. D. and Coggeshall, L. T. Complement fixation in human malaria with an antigen prepared from the monkey parasite, Plasmodium knowlesi, J. Exper. Med. 69:379-398, 1938.

7. Fairley, N. H. A preliminary report on an investigation of the immunity reactions in Egyptian bilharziasis, J. Roy. Army Med. Corps. 32:243, 1919.

8. Gillespie, W. A. The influence of lysolecithin and of incubation on the shape, size, and fragility of erythrocytes, Quart. J. Exper. Physiol. 32:113, 1943.

9. Hewitt, R. I. Studies on host-parasite relationship of untreated infections with Plasmodium lophurae in ducks, Am. J. Hyg. 36:6-42, 1942.

10. Hewitt, R. I. and Richardson, A. P. Direct plasmodicidal effect of quinine, atabrine, and plasmochin on Plasmodium lophurae, J. Infect. Dis. 73:1-11, 1943.

11. Hewitt, R. I., Richardson, A. P., and Seager, L. D. Observations on untreated infections with Plasmodium lophurae in 1200 young white Pekin ducks, Am. J. Hyg. 36:362-373, 1942.

12. Jacobs, H. R. Immunization against malaria Increased protection by vaccination of ducklings with saline-insoluble residues of Plasmodium lophurae mixed with a bacterial toxin, Amer. J. Trop. Med. 23:597-606, 1943. Abstracted in Trop. Dis. Bull. 41:458-459, 1943.

13. Jorpes, Erik. The protein component of the erythrocyte membrane or stroma. Biochem. J. 26:1488-1503, 1932.

14. Kingsbury, A. N. Some investigations of malarial fevers. IV. The complement fixation reaction, Trans. Roy. Soc. Trop. Med. & Hyg. 20:359-364, 1927. Abstracted in Trop. Dis. Bull. 24:746-747, 1927.

15. Kligler, I. J. and Yoeli, M. The diagnostic and epidemiologic significance of the complement fixation test in human malaria, Amer. J. Trop. Med. 21:531-543, 1941.

16. Kolmer, J. A. Technic of kolmer complement fixation tests for syphilis employing 1/5 amounts of reagents, Am. J. Clin. Path. 12:109-115, 1942.

17. Maizels, M. Preservation of orgaine phosphorous compounds in stored blood by glucose, Lancet. 1:722-723, 1941.

18. Maizels, Montague. Phosphate, base and hemolysis in stored blood, Quart. J. Exper. Physiol. 32:143-181, 1943.

19. Maizels, M. and Whittaker, N. Preservation of stored blood, Lancet. 2:1219-1221, 1939.

20. Maizels, M. and Whittaker, N. Effects of storage on human erythrocytes, Lancet. 1:113-116, 1940.

21. Maizels, M. and Whittaker, N. Diluents for stored blood, Lancet. 1:590-593, 1940.

22. Manson-Bahr, P. Researches upon the production of malaria antigen for a complement deviation test, Trans. Roy. Soc. Trop. Med. & Hyg. 21:63-67, 1927. Abstracted in Trop. Dis. Bull. 24:746-747, 1927.

23. Massa, M. Remote eriecenti ricerche sulla devioxione del complemento nei malarici, Pathologica. 21:18-23, 1929.

24. Mollison, P. L. and Young, I. M. In vivo survival in the human subject of transfused red blood cells after storage in various preservative solutions, Quart. J. Exper. Physiol. 31:359-408, 1942.

25. Potaperko, N. A. (Die asmotische resistenz der erythrocyten bei malaria), Russian Jl. Trop. Med. 7:234-239, 1929. Abstracted in Tro. Dis. Bull. 27:215-216, 1929.

26. Schuffner, W. A. P. and Esseveld, H. (Bejdrage tot het vraagstuk der perniciositeit van de laveranie malaria). Genusk. Tydsche. v. Nederl. Indie. 76:3038-3045, 1936. Abstracted in Trop. Dis. Bull. 34:383, 1936.

27. Sintan, J. A. and Ghosh, B. N. Studies of malarial pigment (haemozoin), III. Further researches into the action of solvents, and the results of observations on the action of oxidising and reducing agents, on optical properties, and on crystallisation, Records of the Malaria Survey of India, 4:205-221, 1934.

28. Stratman-Thomas, W. K. and Dulaney, A. D. Immunologic studies in malaria with special reference to the diagnosis of malaria, Amer. J. Trop. Med. 20:717-725, 1940.

29. Thomson, J. G. Preliminary note on the complement deviation in cases of malaria: A new aid to diagnosis, Brit. Med. Jl. 628-629, 1918. Abstracted in Trop. Dis. Bull. 13:87, 1918.

30. Thomson, J. G. Complement deviation in malaria and the question of the influence of malaria on the Wassermann reaction, Trans. Soc. Trop. Med. & Hyg.

13:18-20, 1919. Abstracted in Trop. Dis. Bull. 15:130-131, 1919.

31. Thomson, J. G. Experiments on the complement fixation in malaria with antigens prepared from cultures of malaria parasites (P. falciparum and P. vivax) War office observations on malaria by medical officers of the army and others, 80-94, 1919. (London: H. M. stationery office). Abstracted in Trop. Dis. Bull. 15:421, 1919.