and 6.0 ml of 60% (by volume) sulfuric acid were thoroughly mixed and heated for 20 minutes at 80°C. The intensity of the color in the cooled solution was measured at 520 mμ. For a blank sample, 0.4 ml of the same protein solution was mixed with 0.2 ml of distilled water and 6.0 ml of 60% sulfuric acid and treated as described above. The error of the method is ±2.0%. Determinations were made in duplicate.

Summary. A tabulation of the elementary, (partial) amino acid, and carbohydrate composition of the preparation of C'1 is presented in Table I. The figure for hydroxy amino acid content of the C'1 preparation (23.4%) compares favorably with the value of 21.3% obtained for a β-globulin concentrate by Brand, Kassel, and Saidel,[10] and with the value of 22.5% obtained by these authors for γ-globulin.

15556

Preparation and Properties of Erythrocyte-free Avian Plasmodia.*

LESLIE A. STAUBER AND HARRY A. WALKER. (Introduced by A. P. Richardson.)

From the Bureau of Biological Research, Rutgers University, and the Division of Pharmacology, The Squibb Institute for Medical Research, New Brunswick, N.J.

While studying the serological specificity of avian plasmodia using hemolyzed, parasitized erythrocytes as antigen and the rabbit for the production of antiserum we confirmed the experience of previous workers[1,2] that absorption of such antisera with uninfected erythrocytes seemed to remove all agglutinative or precipitative antibodies reactive with infective erythrocytes or extracts of them. It has been suggested that the malaria parasite may contain substances antigenically similar to normal blood cell components which would explain this phenomenon.[3-5] However, this suggestion does not explain the species and even strain specificity of malarial immunity in the vertebrate host though it may be an additional factor in the reaction. In order to account for the observed absorption of antibodies by uninfected erythrocytes Eaton and Coggeshall reasoned that the complicating factor was "that the parasites cannot be completely separated from the red cells of the monkey or other animals in whose blood they multiply."[1]

Our attempts to produce better test antigens by fragmentation (grinding or freezing and thawing) of duck red cells parasitized with *Plasmodium lophurae* and *Plasmodium cathemerium* were unsuccessful. Microscopically these preparations showed only a small proportion of objects which might be called intact free parasites whether the cells were exposed for a short or a long time to such mechanical treatment. It is possible that such ground materials would be poor test antigens since some precipitin reactions can be demonstrated only after adsorption of the antigen on collodion particles. Thus, the very integrity of the parasite may be essential to the best reactivity if only as a function of the surfaces involved. The removal of the plasma membrane of the erythrocyte in such a fashion as to free whole malaria parasites then seemed the first step in the experimental attack.

The nucleus of the avian erythrocyte served as one of the best clues to the in-

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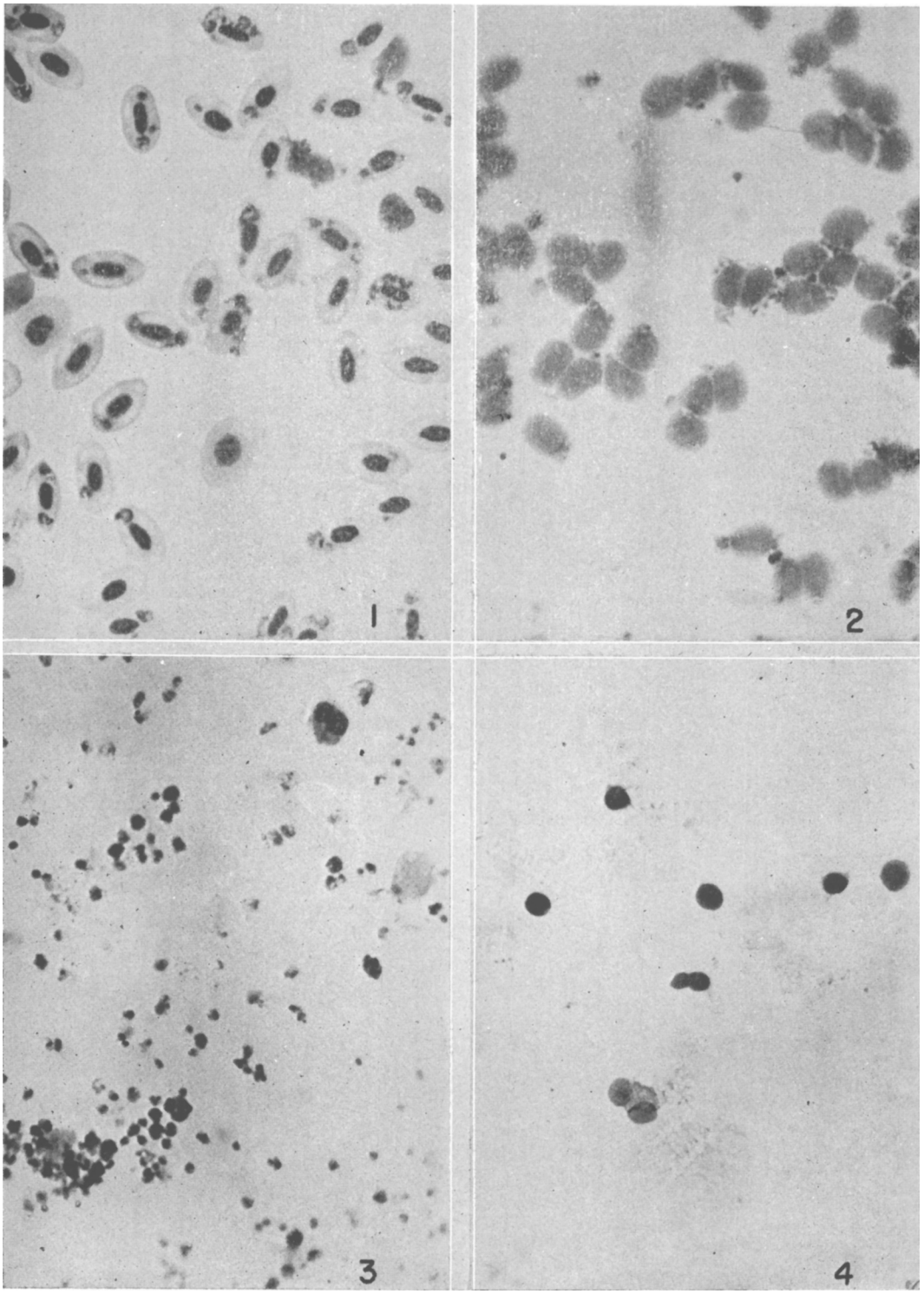

Plate 1.

All figures are dried smears of final preparations fixed in absolute methyl alcohol and stained with Giemsa. We wish to express appreciation to Doctor T. C. Nelson for taking

the photomicrographs. Magnification approx. X1100.

Fig. 1.

Blood of a duck infected with *Plasmodium lophurae* exposed to all steps except hemolysis with saponin.

Fig. 2.

Infected blood exposed to all procedures except those containing enzymes.

Fig. 3.

Free parasites obtained by exposing infected blood to both hemolysis and enzymatic digestion.

Fig. 4.

Smear from end product of treatment of uninfected blood by both hemolysis and enzymatic digestion.

tegrity of the plasma membrane of the erythrocyte since it could be seen very easily in the fresh condition and was readily stainable by methylene blue or by the Romanowsky stains. It was not always possible to demonstrate satisfactorily the plasma membrane of the erythrocyte microscopically. Still, the presence of a parasite close to a nucleus and rigidly maintaining its position relative to that nucleus in a moving fluid medium was clearly indicative of the presence of at least a part of the stroma and was more than suggestive of the presence of the plasma membrane as well.

Although it is clearly indicated in Ponder's[6] summary that hemolysis is not synonymous with fragmentation or lysis of the stroma, the first attempts to free the parasites were made with hemolytic agents. Distilled water, chilled water saturated with CO_2, and various concentrations of NaCl, saponin, and urea were tried. Intact free parasites were not obtained by any of the procedures used. Even a technic successful in freeing *Plasmodium knowlesi*[7] from simian erythrocytes left *P. lophurae* still associated with the red cell nucleus of the duck.

Although living cells resist enzymatic digestion, Northrop[8] showed that intracytoplasmic introduction or proteolytic enzyme caused the disintegration of amebae. During hemolysis a change in the permeability of the erythrocyte occurs such that the large molecule of hemoglobin escapes from the cell.[9] If such altered permeability would permit the penetration of enzymes, cellular fragmentation with release of intact parasites might be accomplished. In the first trials, hemolysis by various agents followed by digestion with trypsin-steapsin mixtures at pH 7.0-7.8 resulted in failure to produce erythrocyte-free malarial plasmodia. However, when the enzyme preparations were tried at pH 5.0-5.5,[10] intact free parasites were obtained.

The method now used is as follows: Sterile parasitized duck blood is drawn into 0.9% saline solution, buffered to pH 7.0[11] and containing 300 mg % of heparin as anticoagulant. One part of the heparinized saline is used for every 19 parts of blood. All equipment and solutions except the saponin are sterile. After centrifugation at approximately 2000 r.p.m. for 10 minutes the plasma is drawn off and the cells washed once with 2-3 volumes of buffered saline. The cells may be washed at this point in the procedure with buffered saline to which formalin has been added in a concentration of 0.2%. In this way approximately 10 ml of packed cells are obtained for every 30-35 ml of duck blood drawn. The washed cells are suspended in 20 × their volume of 1-5000 saponin in buffered saline. The material is then centrifuged for 20 minutes and the supernatant drawn off. The cells are next suspended in 10 × their volume of Seitz-filtered enzyme solution prepared by dissolving 100 mg % of trypsin and steapsin in buffered saline (pH 5.0-5.5). The mixture is centrifuged immediately and the supernatant discarded. This step is necessary to remove any residual saponin. The cells are again suspended in

[6] Ponder, E., *The Mammalian Red Cell and the Properties of Haemolytic Systems*, Berlin, 1934.

[7] Christophers, S. R., and Fulton, J. D., *Ann. Trop. Med. and Parasit.*, 1939, **33**, 161.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1936, **9**, 497.

[9] Davson, H., and Danielli, J. F., *The Permeability of Natural Membranes*, New York, 1943, 263-277.

[10] Ballentine, R., and Parpart, A. K., *J. Cell. and Comp. Physiol.*, 1940, **16**, 49.

[11] Evans, A. C., *J. Infect. Dis.*, 1922, **30**, 95.

10 × their volume of enzyme solution and allowed to stand with occasional shaking at room temperature for a total of 1½ hours, after the first addition of the enzyme. The material is then centrifuged and washed twice with buffered saline and finally made up to a total volume of 10 ml in buffered saline or buffered saline containing 0.2% formalin since with the latter we have found that the preparations keep well for at least several weeks when stored in the refrigerator.

Treated in this way uninfected duck cells lose all visible indications of hemoglobin and appear as a creamy mass of sedimented material in a volume usually equal to less than one-third the volume of packed cells at the beginning of the run. Prepared with erythrocytes heavily infected with *Plasmodium lophurae* (or other species of malaria) the material is muddy in appearance, the depth of the brown color and the volume is dependent upon the degree of parasitization of the erythrocytes.

It is not claimed that the empirical procedures used accomplish much digestion else there would not be the sediment seen with uninfected cells, but microscopically marked changes are evident. In wet mounts only granular material, sometimes in amorphous masses, and leucocytes are seen. It is especially interesting to note that the leucocytes seem entirely unchanged by the methods used. The end product with infected blood contains, in addition to the above, spherical parasites of a range of sizes from slightly more than 1 μ to almost 7 μ in dried smears. Granules of malaria pigment in active Brownian motion can be seen, usually more or less aggregated in a vacuole in one portion of the parasite.

When dried films of such preparations are fixed in absolute methyl alcohol and stained with Giemsa they appear as in Fig. 1-4. All smears were made from similar dilutions of original material and are as nearly comparable as possible. Fig. 1 is a smear of infected cells which have undergone exposure to all the steps except that of hemolysis in saponin. The parasites are practically normal in appearance and are largely contained within duck erythrocytes. Where outside of a cell they are closely adjacent to one of the paler nuclei, presumably belonging to an erythrocyte hemolyzed in the act of making the film. Note that in these cases a plasma membrane is not visible. Fig. 2 is from infected blood exposed to all procedures except those containing enzyme. The typical picture of stained hemolyzed red cells is seen. No plasma membranes are found and slightly swollen, paler nuclei are evident. Parasites are readily seen, usually pressed against the erythrocyte's nucleus and rarely more than a width of a cell away from the nucleus even though almost 50 parasites may be seen in the photomicrograph. Fig. 3 shows the result of hemolysis and digestion of infected duck blood. Aside from the leucocyte in the field and 2 smudges (incompletely altered erythrocytes) the field is full of free, intact malaria parasites. Their morphology is typical of that of the species to which they belong (in this case *P. lophurae*). The nuclei are somewhat larger and paler but the pigment and vacuolated cytoplasm are quite as seen in ordinary blood smears. *Plasmodium cathemerium* prepared in like fashion can be specifically distinguished from *P. lophurae* by the deeper, more homogeneous staining of the cytoplasm. Fig. 4 shows a field from the smear of uninfected blood after both hemolysis and digestion. Only leucocytes and pale smudges (possibly the remains of erythrocyte nuclei) are visible in the field. The leucocytes seem typical of those seen in ordinary thin films, one at least being obviously a heterophile.

Nonformalinized erythrocyte-free *P. lophurae* prepared as described above have been tested for infectivity by repeated intravenous injection of the material into 100 mg ducklings. Each duckling was injected with 1 ml of parasite suspension containing 3 billion parasites on alternate days for a total of 3 days. (Total dose 9 billion parasites per duck). The first injection was given on the same afternoon the concentrate was prepared and as soon after the last step in the procedure as possible. The material was then refrigerated and handled as if it were sterile until the last injection had been made. Only one of the 5 so treated showed parasites. It

died on the 23rd day after the first injection with a parasitemia of 74%. This does not mean that parasites were able to survive the rigors of being freed from the erythrocyte by the means employed. We have repeatedly seen that small blood clots will protect the erythrocytes from hemolysis and therefore from digestion. Perhaps a single microscopic mass of such material could be infective and result in the death noted. We believe it most probable that the treatment destroys all those parasites freed from red cells.

Summary. By combining saponin hemolysis with enzymatic digestion at low pH avian plasmodia have been freed from the enclosing erythrocytes. These free parasites retain their specific morphology so that in stained smears of the erythrocyte-free parasites *P. lophurae* can be distinguished from *P. cathemerium.* It is probable that the treatment used kills the parasites but the potential value in experimental malariology of this new method is not necessarily reduced thereby.

15557

An Antibiotic (Anti-Smegmatis Factor) Produced by an Actinomycete, Specifically Inhibiting Species of *Mycobacterium.**

ALBERT KELNER† AND HARRY E. MORTON. (Introduced by A. N. Richards.)

From the William Pepper Laboratory of Clinical Medicine and the Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Penn.

The crude culture filtrate of an actinomycete (designated as A-82) has been observed to show extreme specificity in its action. It inhibited only species within the genus *Mycobacterium,* particularly the saprophytic species of *Mycobacteria,* and especially *Mycobacterium smegmatis.* This antibiotic will be referred to as the antismegmatis factor. The apparent genus specificity of the antismegmatis factor gives it unusual interest.

The actinomycete, A-82, was isolated from potted greenhouse soil by methods to be described elsewhere.[1] Although the organism resembles *Actinomyces lavendulae,* it differs sufficiently to justify a description.

Elliptical spores are borne in chains. Hyphae are coarse. No spirals were seen. Optimum temperature for growth about 28°C; growth at 14°C but not at 37°C. Peptone and gelatin media are blackened, but otherwise no soluble pigment is formed. Abundant grey growth on nutrient agar with white to grey spores. Thin spreading growth on Czapek medium with pink spores. Moderate growth on glucose asparagin agar with lavender colored spores. Good growth on calcium citrate agar; spores white, with trace of pink. Poor growth on calcium malate agar with white spores. Abundant grey-brown growth on potato with spores white, becoming lavender; the potato is blackened. Milk becomes alkaline with rapid clearing. Slow and variable liquefaction of gelatin. Moderate hydrolysis of starch.

The culture was maintained on the starch, potato, tryptone medium described by Kelner and Morton.[1]

Since *Mycobacterium smegmatis* (Univ. of Penn. strain P-49) was the organism most susceptible to the action of the antismegmatis factor, it was used for assay purposes. *M. smegmatis* was grown in Bacto-nutrient broth with daily transfers to stimulate rapid and diffuse growth. An 18-24 hour broth culture was used for streaking plates. The usually aggregated growth was dispersed by

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† Present address, Carnegie Institute, Cold Spring Harbor, N.Y.

[1] Kelner, A., and Morton, H. E., in press.