creased agglutination could be observed with anti-P and anti-I antibodies. Bovine plasmin, human and bovine thrombin, and α-chymotrypsin did not alter the agglutinability of erythrocytes.

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Mass Isolation of Malaria Sporozoites from Mosquitoes by Density Gradient Centrifugation* (33782)

DAVID H. CHEN AND IMOGENE SCHNEIDER
(Introduced by E. H. Fife, Jr.)

Department of Entomology, Walter Reed Army Institute of Research, Washington, D.C. 20012

Biochemical and immunological studies of malaria sporozoites are hampered by the lack of efficient mass isolation methods. Dissection of the paired salivary glands from individual mosquitoes is tedious and time consuming. Culturing mature oocysts *in vitro* and collecting the sporozoites liberated shortly thereafter does provide greater numbers and cleaner preparations (1, 2). However, with either method the removal must be accomplished manually and this precludes obtaining extremely large numbers.

As an alternative we investigated the technique of density gradient centrifugation. Although frequently employed to separate cellular and subcellular particles, the technique has recently been extended to the isolation of whole cells and organs (3, 4). The success of Rawley *et al.* (5) in using density gradients to separate mammalian erythrocytes infected with different developmental stages of *Plasmodium berghei* indicated that the technique might be applicable to the isolation of the invertebrate stages of plasmodia.

This report describes the mass isolation of *Plasmodium gallinaceum* sporozoites from *Aedes aegypti* by using a sucrose density gradient.

Materials and Methods. Donor mosquitoes had a 70–90% infection rate with a mean oocyst count of 75/midgut. Since infectivity varied with different lots of mosquitoes progress in purification was based on the reduction of mosquito mass with simultaneous increase in sporozoite concentration for each fractionation step. The results of successive steps were viewed throughout the course of each run under a phase contrast microscope as well as with Giemsa stained preparations.

One thousand (2.6–3.3 g) 9–11-day postinfected mosquitoes were subdivided into 10 lots

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for homogenization, each lot in 6 ml (1:20 w/v) of Clements' saline, pH 6.9, in a 50 ml (150 × 25 mm) glass test tube loosely fitted with a solid glass pestle (70 × 20 mm) fused to a glass rod. The 2 mm overall clearance allowed effective homogenization of the mosquitoes without physical damage to the parasites. The temperature of this and succeeding steps was maintained at 4°. The pooled homogenates were filtered through a 200 mesh (65 μ) Dufour bolting silk cloth mounted on a stainless steel screen of a Millipore filter holder assembly. The residue on the filter was rehomogenized in 30 ml of Clements' saline and filtered. The pooled filtrates had a combined residual mass of 580–830 mg. These steps resulted in a 75–78% reduction of the original mass and yielded an estimated 85–90% of the total extractable sporozoites.

The filtrate was divided equally into four 50-ml centrifuge tubes and centrifuged at 200*g* for 5 min in a Sorvall RC-2 refrigerated centrifuge equipped with a HB-4 horizontal rotor. The supernatants were decanted into 4 clean 50-ml tubes and the residues resuspended, pooled in 15 ml of Clements' saline and recentrifuged at 200*g* for 3 min. The latter supernatants were pooled along with supernatants from the initial centrifugation and spun at 1000*g* for 30 min. Both the fatty layer at the surface and the supernatant were discarded. The residues containing most of the parasites were resuspended, pooled in 20 ml of saline and recentrifuged at 1000*g* for 20 min. These sedimentation procedures were estimated to remove an additional 78–80% (450–660 mg) of the debris leaving a residual mass weighing 130–170 mg and containing between 75 to 80% of the total extractable sporozoites.

Buffered sucrose solutions were prepared by mixing the desired concentration of certified ACS sucrose in 30 ml of distilled water in a 100-ml volumetric flask. The sucrose was completely dissolved in a 95° water bath. Upon cooling to 20°, 25 ml of 4 × concentration of Clements' saline was added, and the remaining volume was filled with distilled water.

A linear gradient was prepared by mixing 13 ml each of a 31% (density = 1.132 g/cm^3) and 42% (density = 1.188 g/cm^3) buffered sucrose solutions in a Buchler Universal Density Gradient Former connected to a peristaltic pump operated at a flow rate of 1.5 ml/min. The gradient was formed in a 30-ml Spinco cellulose nitrate tube fitted in an adapter (70 × 28.7 mm) modified from a Nalgene 50-ml polypropylene centrifuge tube. Linearity of the gradients and the refractive indices of the sucrose solutions were determined with a refractometer (λ = 589 mμ at 23°) and converted to density at 20°. The sucrose gradient was equilibrated at 4° prior to use. Two ml of a 15% sucrose suspension of the sporozoite preparation was carefully layered on the top of the gradient and centrifuged at 16,000*g* for 60 min with the brake off. Twenty-six 1-ml fractions were collected by bottom puncture of a cellulose nitrate tube in a Buchler Density Gradient Fractionator. Four ml of Clements' saline was added to each fraction and the mixtures were centrifuged at 2600*g* for 10 min. The supernatants were discarded, each of the residues was resuspended in 0.005 ml of saline, and conventional thin film smears were made and stained with Giemsa.

Results and Discussion. Fifty-five centrifugation runs were made with gradients of varying linearity. Gradients with an 11 point range from 31 to 42% sucrose gave the most consistent results in isolating the sporozoites from peak fractions at or near the center of the gradient. The distribution of sporozoites within two such gradients together with the sucrose percentages and respective densities are shown in Fig. 1. Values for the mean sporozoite density in the peak fraction ranged from 1.156 to 1.158 g/cm^3. Such a fraction contained about 20% of the total sporozoites in the gradient. If fractions 10–16 were combined, a recovery in excess of 80% of the sporozoites from the gradient was obtained. Up to 10^7 sporozoites could be recovered from the peak fraction and approximately 3.6 × 10^7 sporozoites from fractions 10–16. Based on a 1000 mosquito sample, of which 70–90% are infected, the number of

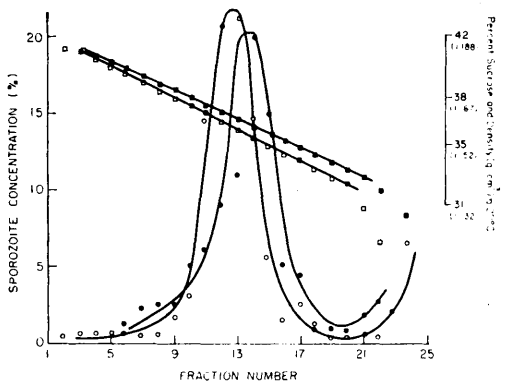

FIG. 1. Distribution of *P. gallinaceum* sporozoites in 1-ml fractions of a 31–42% linear sucrose gradient: sporozoite concentration (○, ●) and sucrose density (□, ■) from two separate runs.

sporozoites recovered per mosquito was estimated at 45,000.

Photomicrographs of sporozoites recovered from peak fractions are shown in Fig. 2a,b. Contaminants in the sporozoite fraction appeared to be transitory, i.e., not stabilized in the gradient, and mostly of mosquito origin. In contrast, the position of the sporozoite band did not change for up to 2 hr of centrifugation. However, centrifugation of longer duration resulted in a gradual loss of resolution. After 4 hr most sporozoites were found at the bottom of the tube, apparently the result of the high osmotic pressure of the sucrose. Consequently, the ideal of isopynic gradient centrifugation of all components was not achieved in the present system and the separation obtained was dependent on both sedimentation rates and relative densities.

The majority of recovered sporozoites were intact and motile although they were no longer infectious to chicks. The latter factor, however, should not be detrimental to their use in most immunological or biochemical studies. It is believed that the loss of infectivity may have been due to the relatively high concentration of sucrose required to obtain the appropriate densities as well as the prolonged time (5 hr) required for a single run. Efforts are being made to minimize these problems by using a different medium such as Ficoll or urografin for the gradient.

This technique is currently being used to isolate sporozoites of a primate malaria, *Plasmodium cynomolgi*, from *Anopheles stephensi* mosquitoes, and efforts are being made to extend its application to other host–parasite systems as well. The relative ease and speed with which large numbers of sporozoites can be obtained by this method should provide added impetus for its use as a preliminary step in a wide range of malaria investigations.

Summary. *Aedes aegypti* mosquitoes, infected with *Plasmodium gallinaceum*, were homogenized and the resulting brei was subjected to a series of filtration and centrifuga-

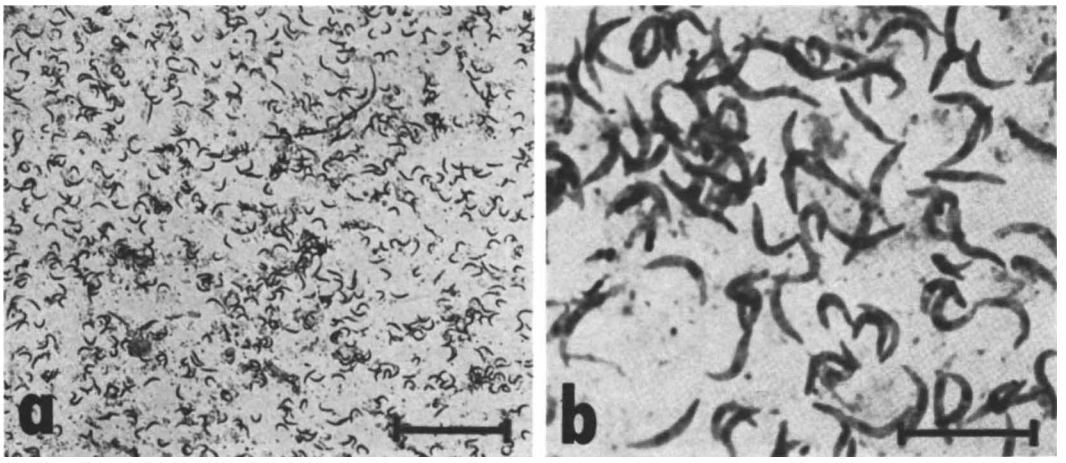

FIG. 2. Appearance of concentrated sporozoites recovered from peak fractions of a sucrose density gradient: Giemsa stained for 2 hr; the line in the lower right hand corner represents 50 μ in (a) and 10 μ in (b).

tion steps prior to being placed on a linear sucrose gradient. The mean density of the sporozoites isolated from the peak fraction was 1.157 g/cm^3 and the number of sporozoites recovered per mosquito was estimated to be 45,000. The technique can readily be extended to the isolation of sporozoites of other plasmodial species and produces a greater yield than is possible by conventional dissection methods.

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Vitamin D Binding Globulin in the Rat: Specificity for the Vitamins D* (33783)

H. RIKKERS, R. KLETZIENS, AND H. F. DELUCA
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

An *in vivo* study of serum protein binding of vitamin D_4-^3H in rats has shown that there exists a specific vitamin D binding globulin (1). In that study there was noted an increasing association of radioactivity with this protein with time. This was associated with the conversion of 60% of the vitamin to the major biologically active metabolite. This increasing association with time could have been due to new protein synthesis or to a more specific binding of metabolite than of parent vitamin. To study this further, the following experiments were performed, comparing the *in vivo* serum protein binding of vitamin D_3-^3H and vitamin D_2-^{14}C in the rat.

Materials and Methods. Tritiated vitamin D. Specifically labeled vitamin D_3-1,2-^3H and randomly labeled vitamin D_2-^{14}C were prepared by methods previously described (2, 3). All vitamin preparations were stored in benzene at 4° until use. No preparation was used which was not 97% chromatographically pure.

Animals. Male weanling rats obtained from Holtzman or Sprague-Dawley companies were fed vitamin D-deficient diet No. 11 as described by Guroff *et al.* (4) for 4–8 weeks.

Experimental procedure. Five to 100 IU of vitamins D_2-^{14}C or D_3-^3H in 0.05–0.1 ml of absolute ethanol were injected intravenously via the jugular vein. Blood samples were obtained from the tail vein at 5, 90, 150, and/or 240 min after injection. Serum was obtained by centrifugation at 2000 rpm within 30 min of drawing. Three 0.1-ml samples of each serum were subjected to disc electrophoresis within 5 hr of drawing, as previously described (1).

Radioactivity within each gel was located by combustion of the separated proteins and subsequent trapping of the ^3H_2O or $^{14}CO_2$, as previously described (5). Radioactivity measurements were performed in a Packard Tricarb liquid-scintillation counter model 3003 equipped with external standardization.

Gels were sliced in the patterns shown in Fig. 1. The radioactivity of the blank (dpm/mm) was multiplied by the total millimeters of each segment and subtracted from the radioactivity found in each segment. A mean value ± SD was calculated for each

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