

# A TECHNIQUE FOR ESTIMATING TOTAL MALARIAL SPOROZOITES IN MOSQUITOES<sup>1</sup>

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**INTRODUCTION.** Pringle (1965) has shown that 9,555 sporozoites may be produced in a single oocyst of *Plasmodium falciparum*. Newly liberated sporozoites circulate with the hemolymph and lodge in enormous numbers in all the organs of the mosquito's body (Wenyon, 1965). Perhaps as few as 2 percent of the sporozoites produced in the oocysts ever reach the salivary glands (Shute, 1945). Those that fail to do so disappear (Huff, 1949), but small numbers may be found in organs other than the salivary glands for as long as 31 days after infection (Oelerich, 1967).

Shute (1937, 1945) and Shute and Maryon (1960) developed a method, based on microscopic subsampling of a volu-

metric sample of salivary gland suspension, for estimating the numbers of malarial sporozoites in the salivary glands of individual mosquitoes. This method was subsequently improved by eliminating the necessity for volumetric sampling (Shute *et al.*, 1965; Pringle, 1966). However, studies on the production and survival of sporozoites during the period of oocyst maturation, sporozoite liberation and sporozoite migration will require a technique for estimation of their numbers in whole mosquitoes.

The purpose of the present work was to adapt the Shute technique to the estimation of sporozoite numbers in whole mosquitoes and to estimate the net increase in sporozoites, per oocyst, over the period from day 8 to day 15 in *Anopheles stephensi* experimentally infected by a naturally occurring case of *Plasmodium falciparum*.

**MATERIALS AND METHODS.** *Anopheles stephensi* Liston, reared and maintained in an insectary at 27° C and 80 percent R.H., were fed on two cases of naturally occur-

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ring *falciparum* malaria in Phra Phutthabat, Saraburi Province, Thailand. Fed mosquitoes were sacrificed for sporozoite estimation on day 14 or 15 post-feed, at which time sporozoite liberation had essentially ended. An experimental design yielding data in the hierarchical ("nested," "within-within") classification was chosen since this design is well adapted to the evaluation of technique (Steel and Torrie, 1960).

**Experiment 1.** The mosquitoes were infected on June 19, 1967, by feeding on a 32-year-old Thai circulating 2,000 gametocytes and 2,100 trophozoites of *P. falciparum* per cu mm of blood. On July 3 (day 14) 13 mosquitoes were individually ground in Tenbroeck tissue grinders containing 0.5 cc of physiological saline solution. Three smears were prepared from each mosquito suspension as follows: Three 0.01 cc drops of suspension were deposited on a microslide with a serological pipette, and each was spread over a roughly circular area approximately 10 mm in diameter with the tip of a No. 2 insect pin. The smears were labeled, air-dried, fixed in methanol and stained with Giemsa.

Sporozoite counting was carried out in the following manner: The microslide for a given mosquito was seated in the mechanical stage of a bright-field microscope, and the ordinates of the upper and lower limits of each smear were read to 0.1 mm with the vernier scale of the mechanical stage. Within each smear, three additional ordinates were selected with a table of random numbers. The oil immersion lens was focussed on the first smear and positioned over the first randomly selected ordinate with the mechanical stage and vernier scale. A traverse along the ordinate and completely across the smear was made, counting each sporozoite encountered in the traverse. Sporozoite counts were recorded separately for each traverse of each smear.

Estimates of the number of sporozoites carried by each mosquito were computed from the counts made in each traverse of

each smear. The diameter of the oil immersion field was determined at 0.18 mm with a stage micrometer. Variation in traverse length was neglected, the areal fraction of a smear sampled in a single traverse being taken as  $0.18/D$ , where  $D$  is the diameter of the smear in mm obtained by subtraction of the ordinates of its upper and lower limits. Mosquito volumes were neglected, the mosquito fraction represented by a single smear being taken as  $0.01/0.5 = 1/50$  mosquito. Under these rules the mosquito fraction sampled in a single traverse was  $(0.18/D) (1/50) = 0.0036/D$  mosquito, and the sporozoite estimate based on a single traverse was  $N(D/0.0036) = 280 \cdot DN$ , where  $N$  is the number of sporozoites counted in the traverse. These estimates were rounded to the nearest thousand sporozoites.

**Experiment 2.** The mosquitoes were infected on August 31, 1967, by feeding on a 12-year old Thai circulating 7600 gametocytes and 10,000 trophozoites of *P. falciparum* per cu mm of blood. In this case a series of 30 oocyst counts was made on day 8 (September 8), in addition to sporozoite estimates for 23 mosquitoes sacrificed for this purpose on day 15 (September 15). Sporozoite estimates were made as in experiment 1 except that 4 smears were made from each mosquito, 2 traverses were made of each smear and the sporozoite estimates were rounded to the nearest hundred sporozoites.

**RESULTS.** Mean sporozoite estimates over all traverses for each of the mosquitoes in experiment 1 were 0.0, 1.3, 2.7, 3.6, 3.8, 5.9, 6.2, 6.3, 8.7, 9.2, 10.4, 12.3, and 18.9 thousand sporozoites. Those for experiment 2 were  $5 \times 0.0$ ,  $4 \times 0.3$ , 0.4,  $4 \times 0.9$ ,  $2 \times 1.0$ , 1.1, 1.2, 1.4,  $2 \times 3.0$ , 5.3 and 14.1 thousand sporozoites.

Analyses of variance of the sporozoite estimates for the positive mosquitoes of experiments 1 and 2 are presented in Table 1. The sensitivity of the design was sufficient to detect differences among mosquitoes in each case. The variation among smears within mosquitoes (experimental error) was large in comparison with the

TABLE 1.—Analyses of variance for estimates of sporozoite numbers in *An. stephensi* infected by 2 cases of *falciparum* malaria

Experiment	Source of Variation	df	SS	MS	F
1	Among mosquitoes	11	2362	214.7	2.81*
	Among smears within mosquitoes	24	1834	76.4	4.52**
	Among traverses within smears	72	1215	16.9	....
	Total	107	5411	....	....
2	Among mosquitoes	17	1466	86.2	9.22**
	Among smears within mosquitoes	54	505	9.3	1.99*
	Among traverses within smears	72	339	4.7	....
	Total	143	2310	...	....

\* Significant at the 95% level.

\*\* Significant at the 98% level.

variation among traverses within smears (sampling error). In consideration of this fact, volumetric sampling (the number of smears per mosquito) was increased at the expense of microscopic subsampling (the number of traverses per smear) in experiment 2, resulting in a considerable gain in efficiency. The standard error of a mosquito mean was 1.8 thousand sporozoites for experiment 1 and 0.4 thousand sporozoites for experiment 2, this depending also on the number of mosquitoes in each experiment.

The numbers of oocysts counted in the 30 mosquitoes dissected on day 8 in experiment 2 were  $5 \times 0^4$ ,  $3 \times 1$ ,  $5 \times 2$ ,  $4 \times 3$ ,  $2 \times 4$ ,  $3 \times 5$ ,  $6$ ,  $2 \times 7$ ,  $11$ ,  $2 \times 16$ ,  $19$  and  $25$  with a mean of 6.2 and a standard error of 1.26 oocysts for the 25 positive mosquitoes. The mean number of sporozoites for the 18 positive mosquitoes used in the sporozoite estimates of day 15 in this experiment, as computed directly from the mosquito means, was 2.0 with a standard error of 0.77 thousand sporozoites. The standard error of the quotient,  $2,000/6.2 = 325$ , is 140, and its 95 percent confidence limits are 30 to 600 sporozoites per oocyst.

DISCUSSION. The strikingly low estimate of the preceding paragraph may reflect either bias in the experimental procedure or a mortality of sporoblasts, oocysts or sporozoites between day 8 and day 15. We have no evidence of bias, but it may be that sporozoites are destroyed in the grinding process, lost in the staining pro-

cedure (Dowling and Shute, 1966) or eclipsed by fragments of mosquito tissue in the finished smear. Deterioration and degeneration of oocysts and sporozoites has been reported by Roubaud (1918), Barber *et al.* (1927), King (1929), Boyd *et al.* (1936), Boyd (1945, 1949), Brumpt (1949) and Russell *et al.* (1963). Since, according to the present report and that of Pringle (1965), the sporozoites to be accounted for amount to  $(100) (9555 - 325) / 9555 = 97$  percent of the total, this matter would appear to be of great importance for further investigation.

CONCLUSIONS. The method employed in this study is imprecise and time-consuming, some 25 hours of microscopy being required for the two experiments. It was nonetheless sufficient to indicate that, at least in some cases, a large proportion of the sporozoite potential represented by the developing oocysts does not materialize.

SUMMARY. An objective method was devised for the estimation of total sporozoite numbers in individual *Anopheles stephensi* mosquitoes infected with the malarial parasite *Plasmodium falciparum*. The standard error of estimates was 1800 sporozoites in one experiment and 400 sporozoites in a second. Application of the method indicated that a large part of the sporozoite potential represented by the developing oocysts is not realized.

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<sup>4</sup>  $5 \times 0 = 5$  zeros (00000), etc.

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## NOTICE

The National Communicable Disease Center announces Course No. 2310-C, Control of Mosquito-Borne Diseases, May 18-22, 1970. The course, directed by AMCA member Paul Rice, is to be conducted by the Vector-Borne Disease Training Unit, Laboratory Division, at the Center's Technical Development Laboratories in Savannah, Georgia, in cooperation with AMCA member Herb Schoof and his associates at TDL, and Oscar Fultz and his staff at the Chatham County Mosquito Control Commission.

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