

Title: Anopheles and Malaria

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Objectives This study is concerned with the identification of those species of Anopheles responsible for transmission of human malaria in Southeast Asia and the determination of the basic factors which affect their vector capability. Specific factors being studied include longevity, host preferences, susceptibility to infection with the various Plasmodium species, and the physiology of blood meal digestion.

Progress The Retention of Chloroquine by Anopheles mosquitoes. Objective It was the purpose of this study to determine whether or not Anopheles mosquitoes could, after ingestion of a blood meal containing the anti-malarial, chloroquine, retain any of this drug.

Background. The use of the mosquito as a screening tool in the mass testing of anti-malarial drugs is currently under investigation (Gerberg et al., 1966). In the mosquito, several drugs have been shown to prevent or interrupt sporogony of some species of Plasmodium (Lumsden and Bertram, 1940a; Lumsden and Bertram, 1940b; Terzian, 1947; Whitman, 1948; Johnson and Aikins, 1948; Geigy and Rahrn, 1949; Terzian and Weathersby, 1949; Terzian et al., 1949; Terzian et al., 1951; Singh et al., 1953; Narayandas and Ray, 1954), however, little attention has been given to the interaction between the drug tested and the mosquito. Whitman (1948) reported that 50 and 25 per cent of a dose of hydroxynaphthoquinone was still present in Aedes aegypti, 24 and 48 hours, respectively, after ingestion.

Methods Randomly selected female Anopheles stephensi from a colonized strain were used in this study and were fed through the membrane feeder described by Rutledge et al. (1964). The mixture fed was prepared as follows: About 2 mg of chloroquine-3-C<sup>14</sup> (specific activity, 2.76 millicurie per millimole; New England Nuclear Corp., Boston, Mass.), accurately weighed to the nearest microgram on a Cahn "Gram" Electrobalance, was dissolved in 0.2 ml, 0.1 N HCl. To the chloroquine solution was added 4.2 ml of Tris-buffered Ringer's solution (Tris buffered-Ringer's solution: NaCl, 9.0 gm; KCl, 0.4 gm; CaCl<sub>2</sub>-6H<sub>2</sub>O, 0.25 gm; NaHCO<sub>3</sub>, 0.2 gm; Dissolved in and made to 1 liter with 0.05 M Tris-HCl buffer, pH 7.5 at 27°C.) containing 614 mg ATP and 1.6 gm sucrose. Four ml of this solution were added to 17 ml of fresh heparinized Rhesus (Macaca mulatta siamica) monkey blood and the mixture was incubated for 1 hr at 37°C in a shaking water bath, to permit interaction of the drug with the blood constituents, prior to being placed in the feeder.

The mosquitoes were allowed to feed for one hour after which they were anesthetized with diethyl ether and the fed mosquitoes removed. After reviving, the fed mosquitoes were randomly distributed into holding cups (20 mosquitoes/cup). On day 0 and each succeeding day, the mosquitoes in one cup were analyzed for their C<sup>14</sup> content. Where mortality occurred, the dead mosquitoes were removed and the original number restored from a pool of fed mosquitoes established at the beginning of the experiment for replenishment purposes. During the experimental period the mosquitoes were held in an insectary at 80°F and 80% relative humidity. For the analysis of their chloroquine-3-C<sup>14</sup> content, the mosquitoes were killed with chloroform and individually weighed on a Cahn "Gram" Electrobalance. The chloroquine was extracted by placing a single mosquito, 0.5 ml H<sub>2</sub>O, 0.5 ml conc. NH<sub>4</sub>OH, 4 ml heptane, and a small amount of broken glass in the homogenizing cup of a Sorvall "Omnimixer" Micro Homogenizer and blending at top speed for 2 min at 0°C. After homogenizing, the mixture was transferred to a screw-cap culture tube, centrifuged to enhance layer separation, and 2 ml of the heptane layer transferred to a stainless steel

planchet for counting in a Beckman "Low-Beta" geiger counter. Five—5  $\mu$ l samples of the original feeding solution were also extracted, as described above, on day 0 to provide information on the count rate of the feeding solution. All samples were considered to be infinitely thin and were counted for a total of 2000 counts (counting error < 5%).

From the net count rate and chloroquine content of the feeding solution and the net count rate of the mosquito extract, the amount of chloroquine remaining in the mosquito after a given time per mg mosquito was calculated so as to provide comparability between experiments 1 and 2. Weight data on the mosquitoes are provided for completeness (Table 1).

Table 1. Weights of the Anopheles stephensi mosquitoes used in the study of the retention of ingested chloroquine— $^3\text{C}^{14}$ .

Day	Mosquito wght. (mg) (Ave. $\pm$ S.D.)	
	Exp. 1	Exp. 2
0	1.588 $\pm$ 0.34	1.804 $\pm$ 0.54
1	1.391 $\pm$ 0.36	1.679 $\pm$ 0.40
2	1.197 $\pm$ 0.36	1.418 $\pm$ 0.36
3	1.006 $\pm$ 0.20	1.309 $\pm$ 0.34
4	1.164 $\pm$ 0.32	1.365 $\pm$ 0.32
5	1.322 $\pm$ 0.43	1.251 $\pm$ 0.22
6	1.184 $\pm$ 0.14	1.166 $\pm$ 0.23
7	1.087 $\pm$ 0.30	1.376 $\pm$ 0.30

Results and Discussion It was found that during the first 2 days of the experiment, approximately 90 per cent of the ingested chloroquine was lost (Figure 1). From day 3, postfeeding, to the end of the experiment (7 days post-feeding), the amount of chloroquine remaining per mg mosquito tended to decrease slowly with an average of 6.8 per cent of the ingested dose still present in the mosquito upon termination of the experiment. Translation of this amount to that retained by a mosquito consuming a 1  $\mu$ l blood meal from a human taking a prophylactic dose of chloroquine (Assuming a level of 100  $\mu$ g/liter whole blood) indicates that about 0.009 m $\mu$ g of chloroquine would still be in the mosquito after 3 days (9% of the ingested dose was found in the mosquito after this time period).

At present, nothing is known about the site or sites of storage of the retained chloroquine nor about the chemical form, unchanged or metabolically altered, which is retained.

Johnson and Akins (1948) have shown that continuous maintenance of Aedes aegypti on 2% sugar solution containing 1 gm per liter chloroquine did not prevent development of the malaria parasite, Plasmodium gallinacuem. However, the stability of the drug in sugar solution, the time and frequency of feeding on the drug, the volume ingested, its concentration in the mosquito, and its effects on the biochemical development of the parasite under these conditions were not determined.

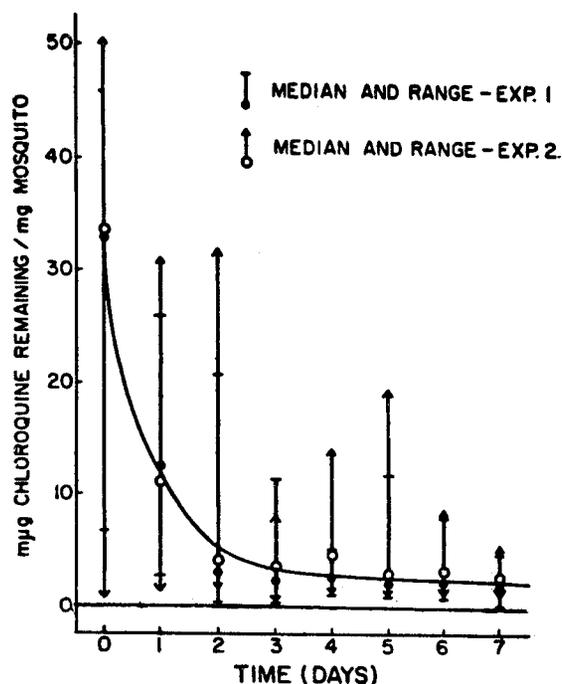


Figure 1. The retention of chloroquin-3-C<sup>14</sup> by Anopheles stephensi as a function of time (days) post-feeding.

Schellenberg and Coatney (1961) have noted that  $10^{-5}$  M chloroquine inhibited the incorporation of P<sup>32</sup> into the DNA and RNA of P. gallinaceum, in vitro. It was subsequently shown that there was an interaction between chloroquine and DNA (Cohen and Yielding, 1965a; Allison et al., 1965). Cohen and Yielding (1965b) have demonstrated that  $10^{-5}$  M Chloroquine inhibited DNA formation in Escherichia coli and that the inhibition was due to blocking of the DNA polymerase reaction. Based on a chloroquine content of 0.009 mug, the concentration in the mosquito would be about  $3 \times 10^{-8}$ M at the end of three days, a level which is below that found to inhibit DNA polymerase (Cohen and Yielding, 1965b).

Degeneration of established oocysts in the mosquito is only rarely observed, and it appears that the critical period for survival of the parasite is during the first three days following ingestion. In addition to the metamorphoses and migrations required of the parasite during this period, there is extensive nuclear activity. Besides the divisions and fusions involved in microgametogenesis and fertilization, there are post-zygotic meiotic and mitotic divisions in the early oocyst (Bano, 1959). The chloroquine concentration in the mosquito is greatest during this period and conditions would be favorable for the expression of any mutagenic or biochemically selective properties that chloroquine might possess.

It is widely believed that the prophylactic use of chloroquine has contributed to the rise of chloroquine-resistant strains of Plasmodium falciparum. We have demonstrated that some of this drug is retained in the tissues of Anopheles stephensi, and it is possible that pharmacological pressure by such retained chloroquine in the mosquito phase of parasite development, could have contributed to the acquisition of resistance by P. falciparum.

Summary Anopheles stephensi, fed on Rhesus monkey blood containing the antimalarial drug, chloroquine-3-C<sup>14</sup>, were found to retain some of the drug for at least 7 days. The possible relationship of the exposure of sporogonic stages of malaria parasites to low levels of chloroquine in the mosquito to the development of drug resistant strains of malaria is raised.

Experimental Infection of Anophelines with human malaria. During the period of this report, laboratory reared anopheline mosquitoes were fed on 15 human cases of falciparum malaria, 5 of vivax and one of mixed falciparum-vivax malaria. Routine searches of the stomachs of mosquitoes for the ookinete stages of the malarial parasite were made in each of these experiments. It was apparent that post-zygotic mortality is a significant factor in malarial cases that are non-infective for mosquito, because of the high proportion of mosquitoes in the above experiments in which development failed to proceed from the ookinete to the oocyst stage. Individuals within groups of like mosquitoes fed simultaneously on the same gametocyte carriers varied widely with respect to the number of oocysts that subsequently developed. However, no major differences in susceptibility within mosquitoes of the same species and strain have been identified.

A convenient technique for estimating the total number of sporozoites carried by single mosquitoes was developed. The technique is based on random transect sampling of a stained smear prepared from a measured droplet of a suspension of the infected mosquito ground in a measured volume of diluent. The diameter of the microscope field and dimensions of the smear were also measured. This procedure is expected to yield information on the sporozoite load of individual mosquitoes and the number of sporozoites inoculated by the bite of a single mosquito. In 12 Anopheles stephensi infected by a falciparum gametocyte carrier, total sporozoite loads ranged from 1.3 to 18.9 thousand sporozoites with a standard error of 1.8 thousand.

An effort was made during this report period to compare the susceptibilities of several species of anophelines to the strains of the malarial parasites prevalent in Central Thailand. The results of these comparative feedings are summarized in Table 2.

Table 2. Comparative feedings of anopheline species on human malaria cases.

Dissection Results							
Mosquito "A"					Mosquito "B"		
Parasite	No. of Cases	Species	No. Diss.	Per Cent Pos.	Species	No. Diss.	Per Cent Pos.
<u>P. falciparum</u>	1	<u>A. balabacensis</u>	5	60	<u>A. maculatus</u>	1	0
	3	<u>A. balabacensis</u>	15	7	<u>A. minimus</u>	8	0
	6	<u>A. balabacensis</u>	42	24	<u>A. stephensi</u>	106	19
	1	<u>A. balabacensis</u>	19	11	<u>A. vagus</u>	2	0
	1	<u>A. stephensi</u>	8	25	<u>A. maculatus</u>	2	0
	1	<u>A. stephensi</u>	10	0	<u>A. subpictus</u>	3	0
	4	<u>A. stephensi</u>	123	21	<u>A. vagus</u>	22	18
	1	<u>A. subpictus</u>	3	0	<u>A. vagus</u>	9	0
	<u>P. vivax</u>	1	<u>A. balabacensis</u>	21	0	<u>A. maculatus</u>	1
1		<u>A. balabacensis</u>	8	0	<u>A. minimus</u>	3	0
7		<u>A. balabacensis</u>	44	39	<u>A. stephensi</u>	92	10

### Publications

1. Langer, B.W., Rutledge, L.C. and Gould, D.J., 1968. Chloroquine: Retention by Anopheles stephensi after ingestion in a blood meal. Mosquito News (Submitted for publication).
2. Rutledge, L.C., Gould, D.J. and Tantichareon, P., Factors affecting the infection of Anophelines by Plasmodium falciparum in Thailand. (In preparation)
3. Rutledge, L.C., Gould, D.J. and Tantichareon, P. Factors affecting the infection of Anophelines by Plasmodium vivax in Thailand. (In preparation)

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