CHLOROQUINE: RETENTION BY *ANOPHELES STEPHENSI* AFTER INGESTION IN A BLOOD MEAL

BERNHARDT W. LANGER, JR., LOUIS C. RUTLEDGE AND DOUGLAS J. GOULD

Departments of Parasitology and Medical Entomology, Medical Research Laboratory, SEATO Medical Project, Rajavithi Road, Bangkok, Thailand

or

SEATO Medical Project, U. S. Component, APO—San Francisco, 96346

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 Rahm, 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 percent of a dose of hydroxynaphthoquinone was still present in *Aedes aegypti*, 24 and 48 hours, respectively, after ingestion.

This communication presents data on the retention of chloroquine-3-C^{14} (7-chloro-4 (4′-diethylamino-1-methylbutylamino) quinoline), after ingestion as part of a blood meal by *Anopheles stephensi*.

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^{14} (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; $CaCl_2$-$6H_2O$, 0.25 gm; Na HCO_3, 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 "O" and each succeeding day, the mosquitoes in one cup were analyzed for their C^{14} 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 percent relative humidity.

For the analysis of their chloroquine-3-C^{14} 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_2O, 0.5 ml conc. NH_4OH, 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, centri-

fuged to enhance layer separation, and 2 ml. of the heptane layer transferred to a stainless steel planchett for counting in a Beckman "Low-Beta" geiger counter. Five μl samples of the original feeding solution were also extracted, as described above, on day "O" 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 2,000 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–C^{14}.

Day	Mosquito wght. (mg) (Ave.±S.D.)	
	Exp. 1	Exp. 2
0	1.588±0.34	1.804±0.54
1	1.391±0.36	1.679±0.40
2	1.197±0.36	1.418±0.36
3	1.006±0.20	1.309±0.34
4	1.164±0.32	1.365±0.32
5	1.322±0.43	1.251±0.22
6	1.184±0.41	1.166±0.23
7	1.087±0.30	1.376±0.30

The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed throughout this study.

It was found that during the first 2 days of the experiment, approximately 90 percent of the ingested chloroquine was lost (Fig. 1). From day 3, post-feeding, 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 percent 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 μl blood meal from a human taking a prophylactic dose of chloroquine (assuming a level of 100 μg/l whole blood) indicates that about 0.009 mμg of chloroquine would still be in the mosquito after 3 days (9 percent 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. Further investigations are planned in this area.

Johnson and Aikins (1948) have shown that continuous maintenance of *Aedes aegypti* on 2 percent sugar solution containing 1 gm per liter chloroquine did not prevent development of the malaria parasite, *Plasmodium gallinaceum*. 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

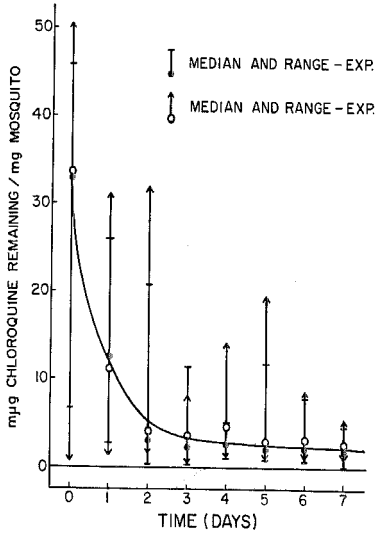

FIG. 1.—The retention of chloroquine–3–C^{14} by *Anopheles stephensi* as a function of time (days) post-feeding.

under these conditions were not determined.

Schellenberg and Coatney (1961) have noted that 10^{-5} M chloroquine inhibited the incorporation of P^{32} 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 mμg, the concentration in the mosquito would be about 3×10^{-8}M at the end of 3 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 3 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^{14}, 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 discussed.

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