PLASMODIUM GALLINACEUM RELEASE FROM CHICKEN ERYTHROCYTES BY ULTRASOUND[1]

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Abstract. Erythrocytes of chickens with *Plasmodium gallinaceum* infection were exposed to ultrasound at frequencies between 334 and 857 kHz. All frequencies studied caused erythrocyte disruption and release of free parasites if the intensity was great enough, although irradiation with 334 kHz appeared to cause more rapid erythrocyte disruption at comparable intensities than did irradiation at the other frequencies studied. Cellular disruption was slower in plasma than in saline, Alsever's solution, or phosphate buffer. Any intensity which caused cavitation caused disruption at a rate roughly proportional to the intensity. When cavitation was suppressed, disruption was almost stopped. The probable sequence of events during sonication of erythrocytes was local damage to the membrane resulting in distortion of the erythrocyte followed by subsequent hemolysis leaving the parasite and erythrocyte nucleus enclosed in the ghost. On further sonication the membrane disrupted and both parasite and nucleus were released. If the parasites were not removed from the ultrasonic field, they, in turn, were disrupted.

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Much experimentation has been undertaken to develop effective procedures for isolating malaria parasites from their host cells. The low degree of reproducibility of the procedures, the failure of most lytic procedures to break the erythrocyte membrane and free the parasite from the stroma (Sherman 1964a), the physical or chemical alteration or loss of antigenic components of the parasite concomitant with the separation (Tobie 1964; Diggs 1964), the destruction of the parasites during the extraction procedure, the contamination of the separated antigens by the reagents used for erythrocyte lysis, (Zuckerman 1964) and the low order of specificity and sensitivity of the serologic tests employing the antigens obtained (Tobie 1964; Sherman 1964b) are among the difficulties encountered with extraction procedures. Ultrasonic disruption of the erythrocyte has been used previously as a method for freeing malaria parasites from the host erythrocytes (Verain and Verain 1956; Kreier *et al* 1965; Rutledge and Ward 1967; Prior and Kreier 1972a). Some investigators, however, have questioned the use of ultrasound for extraction of blood parasites (Bowman *et al* 1960; Zuckerman 1964).

The use of a range of frequencies of ultrasound in the release of blood parasites has not been thoroughly investigated, possibly because most biologists do not have access to variable frequency ultrasonic generators. Appropriately controlled ultrasonic energy may be of considerable use for selectively breaking cells. Certain ultrasonic frequencies exist at which specific cell types are reported to be ruptured more rapidly than at other frequencies (Ackerman 1962). The present investigation was undertaken to determine the frequency of ultrasonic energy, intensity, and time of exposure which would be optimal for the breakdown of host erythrocytes and release of malarial parasites.

MATERIALS AND METHODS

White Leghorn chickens were used as experimental animals. The *Plasmodium gallinaceum* strain was maintained by transfer of blood to susceptible chickens at the time the chickens were bled to obtain erythrocytes for the experiments. Parasitemia at the time of collection ranged from about 20% to 80%. Heparin or

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EDTA was used to prevent coagulation of freshly drawn blood.

Per cent packed erythrocytes in fresh and ultrasonicated specimens was determined by the microhematocrit method and blood films were prepared and stained by the Giemsa method for microscopic observation. Unstained preparations were prepared as wet mounts for examination by phase-contrast microscopy. Percentages of the various cell types were determined on the wet preparations and the stained films by standard differential counting procedures. Hemoglobin release was determined by the cyanomethemoglobin method on the supernatant fluid of irradiated specimens after centrifugation to remove the nonlysed erythrocytes.

The ultrasonic oscillator was designed and assembled by the Ultrasonics Laboratory of the Department of Welding Engineering, The Ohio State University. It consisted of a jacketed irradiation chamber filled with distilled water for sound transmission from the transducer crystal to the tube containing the specimen. The jacket was filled with a crushed ice-methyl alcohol mixture for cooling during tests. The bottom of the irradiation chamber contained the transducer housing which held and permitted easy access to the various lead zirconate-lead titanate transducer crystals used (Piezoelectric Division, Clevite Corp.). The specimen container was sealable and in experiments where cavitation was to be suppressed, compressed air was pumped into the container so that no cavitation occurred. Specimens were held in thin walled polypropylene centrifuge tubes, selected because tests showed that they neither reflected nor absorbed ultrasonic energy to any significant degree. Cellulose nitrate, nontempered glass, polyethyele and pyrex tubes were tested and discarded because they reflected or absorbed ultrasonic energy to a high degree (Weiss 1967). An Acoustica model GU 400 variable frequency ultrasonic generator was used to drive the piezoelectric transducer crystals. This unit supplied a maximum of 400 watts of power to the transducers over a frequency range of 8 to 1200 kHz. The driving voltage was monitored with a Hewlett-Packard 4100 voltmeter and the frequency was monitored with a Hickok digital system, models DP 150 and DMS 3200. For details of the construction of the oscillator, see Weiss (1967) and Prior (1968).

Acoustic intensity in the specimen container was determined by immersion of a barium titanate receiving probe in the specimen and direct measurement of intensity. As all such probes are frequency dependent in their response, intensity measurements could not be compared from one frequency to another. We also estimated acoustic intensity by measuring the height of the fountain at the sample surface. The intensities used are referred to as levels I, II, III, and IV which produced fountains about 0.64, 1.9, 3.2 or 5.1 cm (0.25, 0.75, 1.25 or 2.0 in.) in height. Intensity levels were located for each of the frequencies studied (334–347, 515–518, 535–550, 572–589, 820–857 kHz) at which complete hemolysis and fragmentation of the erythrocyte membrane, with release of free parasites, occurred.

RESULTS

The hemolytic and disruptive action of ultrasound was influenced by whether or not cavitation was present in the test system (fig. 1). With ultrasonic fields of comparable energy, the process of erythrocyte breakdown was retarded by suppression of cavitation. Yields of free parasites were also reduced by suppression of cavitation. The effects of ultrasound were progressive with time. Parasites were freed by the action of ultrasound on the erythrocyte but if they were not removed from the field, they, in turn, were disrupted.

In the study shown in figure 1, maximum accumulation of parasites occurred in 4 minutes. Each of the suspending media tested (phosphate buffered saline, Alsever's solution, Ringer's solution and plasma) permitted cellular disruption to proceed but the rate was slower in plasma than in the other three media.

The breakdown of *Plasmodium gallinaceum*-infected erythrocytes, with release of free malaria parasites, was found to decrease with increasing erythrocyte concentration. During the 10 minute exposure period, 25% and 50% suspensions of erythrocytes were not significantly disrupted but the 5% suspension was completely lysed in 4 minutes. Subsequent studies indicated that erythrocytes in 10% and 15% suspensions were lysed at about the same rate as erythrocytes in 5% suspensions, other factors being equal, while erythrocytes in 20% concentration were disrupted more slowly.

All of the intensity levels tested caused breakdown of erythrocytes with release of parasites (fig. 2). At the lower intensity levels (I and II), the rate of breakdown was slow, and a large parasite accumulation did not occur because the rate of release was low and roughly equal to the subsequent rate of disruption. At the two higher intensity levels (III and IV), there were peaks in free parasite concentration. Exposure beyond these optimums reduced the yield. With the highest intensity used (level IV), the peak came earlier than with the next highest intensity (level III). The most rapid breakdown of erythrocytes and optimum

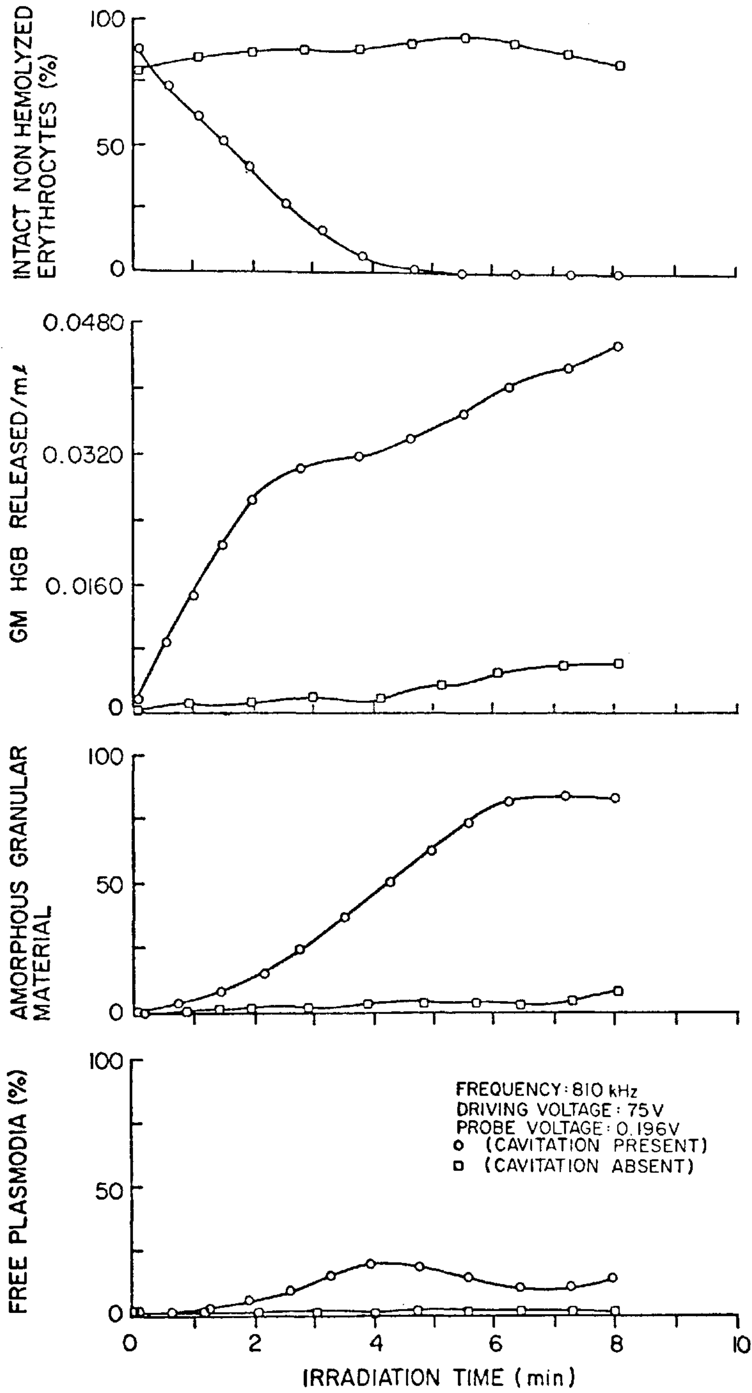

FIGURE 1. Effect of suppression of cavitation on release of plasmodia from erythrocytes, showing retardation of erythrocyte breakdown and reduced yields of free parasites.

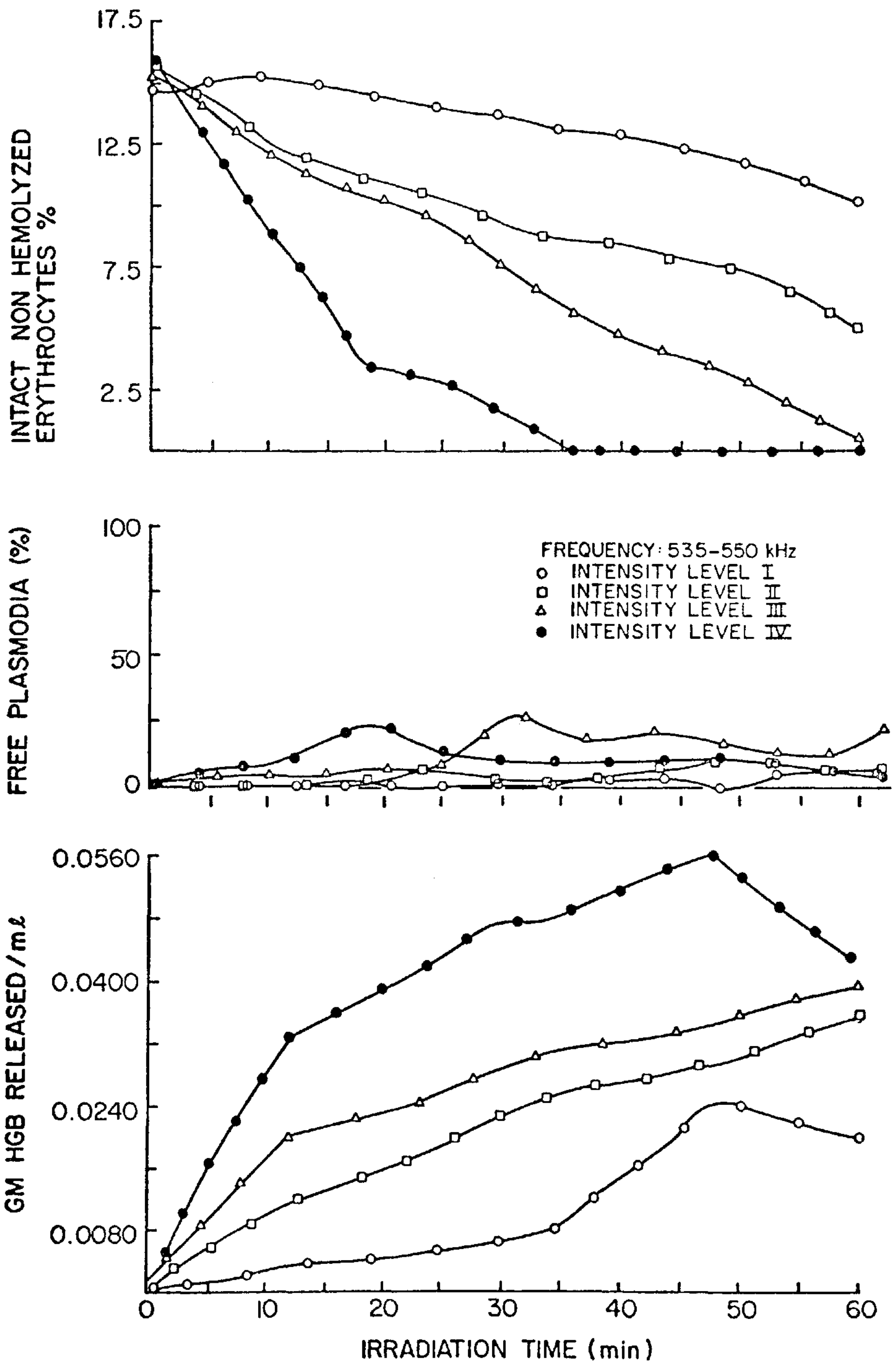

FIGURE 2. Relation of intensity of sonic energy to release of plasmodia from erythrocytes. Rate of erythrocyte breakdown was proportional to intensity of sonic energy. Only at the higher intensities was there significant accumulation of free parasites.

yield of free parasites occurred, at roughly comparable intensities at the lowest frequency tested, 334–347 kHz (table 1).

DISCUSSION

Several investigators have shown that at certain frequencies, cells of a particular type are ruptured more readily than at other frequencies (Ackerman 1952; Binstock 1960). The efficient disruption of chicken erythrocytes by 334–347 kHz ultrasonic fields may indicate that this frequency has a cellular resonance in the chicken erythrocyte.

Lehmann (1953) indicated that, "the acoustic intensity necessary to cause cavitation is proportional to frequency." The rupture of cellular envelopes by ultrasonic irradiation has been ascribed to the large local shearing stresses produced in the vicinity of the rapidly expanding and contracting cavitation bubbles (Ackerman 1953; Grabar 1953; Hughes and Nyborg 1962). We observed that suppression of cavitation stopped erythrocyte disruption. This observation supports the cavitation theory of disruption. It is possible that in our studies, more intense cavitation occurred with comparable energy input at 337 kHz than at the other frequencies tested and that this factor, not resonance, was responsible for the effect we observed.

Ultrasound at all the frequencies tested caused progressive disruption of *Plasmodium gallinaceum*-infected chicken erythrocytes. Rutledge and Ward (1967) reported that the final result of continued sonication, at effectual frequencies was complete disruption of all organized structure. Sonication at the appropriate intensity and for the appropriate time for the system being used appears more important than does the frequency of the ultrasonic waves, even though the lower frequency we tested (about 337 kHz) did, other factors being equal, disrupt the erythrocytes more efficiently. Experiments using a continuous-flow system at 10 kHz with mammalian erythrocytes infected with *P. berghei* have produced morphologically intact viable and antigenically pure parasite preparations (Prior and Kreier 1972a; Prior and Kreier 1972b; and Prior *et al* 1973).

Batch sonication was used in our present study. At the times selected as optimal for release of free parasites, intact erythrocytes and amorphous debris were usually present in the preparation. It is known that not all erythrocytes in the container were exposed to ultrasonic

TABLE 1
Relation of frequency of a moderately intense ultrasonic field to the disruption of erythrocytes of chickens infected with Plasmodium gallinaceum.

Parameter Measured	Sonication (kHz)	Time of Irradiation (min.)					
		0	12	24	36	48	60
Intact RBC remaining (Hematocrit)	334–347	15	10	7	0	0	0
	515–518	15	15	15	14	13	12
	535–550	16	12	11	9	8	5
	572–589	15	12	10	10	8	7
	820–857	16	14	14	13	12	10
Hb release (mg/ml)	334–347	1.6	17.6	45.2	42.0	46.8	46.0
	515–518	2.4	5.2	6.8	12.0	12.8	11.2
	535–550	0.0	11.2	17.6	26.0	29.2	35.2
	572–589	0.0	1.2	1.2	1.2	2.2	2.4
	820–857	0.8	0.7	1.0	1.4	1.9	2.4
% Parasites released	334–347	1	4	2	41	29	21
	515–518	0	3	1	1	8	2
	535–550	0	0	5	1	7	4
	572–589	0	2	2	7	8	7
	820–857	2	0	5	6	7	6

energy to the same degree and that parasites freed early in the sonication period were later disrupted.

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