

Evaluation of In vitro Drug Sensitivity of Human Plasmodium falciparum
by Incorporation of Radioactive Isoleucine

Principal Investigators: Peter K. Iber, MAJ, MSC
Katchrinnee Pavanand, M.D.

Associate Investigators: Norman E. Wilks, LTC, MSC
Edward J. Colwell, LTC, MC

OBJECTIVE: This investigation was an attempt to assess chloroquine sensitivity of P. falciparum isolated from naturally infected Thai nationals by employing both the in vitro culture system described by Diggs et al and the protein incorporation of C^{14} isoleucine as a measure of plasmodial growth. If successful, such a system could be adapted to the field to detect resistant malaria in less than the 28 day observation period prescribed by WHO⁸.

BACKGROUND: Cultivation of malaria parasites in vitro has been restricted generally to experimentation with animal plasmodia. Despite the successful culture of Plasmodium falciparum as early as 1912¹, the culture of native falciparum malaria for extended periods of time has been hampered by a lack of knowledge of parasite metabolism and biochemical requirements from the host red cell. Siddiqui et al² reported successful culture for 48 hours but only on rare occasion did they achieve multiplication rates where the number of parasitized cell greatly increased after schizogony. More recently, Diggs and associates³ have reported a culture system in which five to eight fold increase in parasitized cells were observed repeatedly and growth periods of greater than 72 hours were routine through the use of media T-199 and heat treated human AB serum. The parasite multiplication was determined by the use of fetal cells as markers for merozoite penetration. By virtue of the increase in survival time and parasite populations this system lends itself to extensive research opportunities including metabolic and pharmacologic studies of Plasmodium falciparum.

Polet and Conrad⁴ have reported isoleucine as an essential amino acid for P. knowlesi. Canfield et al⁵ have described an in vitro technique for testing potential antimalarial drugs using methyl C^{14} methionine incorporation into parasite protein as measurement of drug effect. McCormick⁶ was successful in using C^{14} -isoleucine as a growth marker for P. falciparum in the test described by Rieckmann⁷.

DESCRIPTION: The basic procedures for cultures of Plasmodium falciparum were those previously described³. For drug sensitivity studies the following modifications were necessary.

Parasites: Patients from the Yala Provincial Hospital and the Malaria Eradication Center, Yala, Thailand were used as donors for parasites of Plasmodium falciparum. Only patients with parasitemias greater than 0.1% were accepted. The blood was collected in a heparinized syringe and the cells were later separated from the plasma by centrifugation at $210 \times G$ for 10 minutes. The cells were then washed twice with physiological saline, centrifuging each time at $210 \times G$ for 10 minutes.

Media: The packed cells from the second washing were then placed into media prepared for each row of a microtitration plate as follows:

Media T-199	0.6	ml	
Heated Serum (AB Group)	0.6	ml	
Washed packed cells	0.125	ml	
C ¹⁴ -Isoleucine*	0.050	ml	(0.048 ug = 2.21×10^5 dpm)
Total volume	1.375	ml	(enough for 6 wells)

The media-cell suspension was then distributed into the wells of one row in the microtitration plate in the array of drug concentrations shown in Table 1 (each well contained 0.2 ml of the media-RBC suspension).

Harvesting Cultures: The culture suspension was quantitatively transferred from each well in one column at the harvest time into a 13x100 mm test tube. A few milliliters of normal saline was needed to effect quantitative transfer. The suspension was centrifuged at 6000xG and the supernatant was discarded. Packed cells were washed twice with 5 milliliters of normal saline, and centrifuged as above, and then 1 ml of 10% trichloroacetic acid was added, mixed well, and centrifuged at 10,000 x G. The supernatant was removed, the precipitate was washed with 1 ml of ethanol-ether (1:1), mixed well, centrifuged, and the supernatant was discarded. Three drops of 30% hydrogen peroxide were added, mixed and incubated at 56°C for 15 minutes. Two milliliters of Soluene (TM 100 Packard) was added to the precipitate which was then incubated 30 minutes at 60°C and transferred to a counting vial using fluor as a rinsing agent for the test tube. The final volume was 12 milliliters.

Drug growth studies were performed using two series of chloroquine concentrations. The first series, covering Yala patients Y-36 to Y-48 (See Table 2), was cultured utilizing a drug concentration of 8 millimicromole chloroquine base per ml blood⁷. This concentration was based on a 12 hour serum level in Thai volunteers (Figure 1) and the partition ratio RBC: Serum of approximately 4:1 to 5:1.

With the 8 millimicromole concentration of chloroquine, although pharmacological, it is difficult to evaluate the culture results. Since the 12 hour serum concentration is transient, it was decided that the 72 hours serum concentration was more realistic for the culture period. Therefore, we revised the chloroquine concentrations in the subsequent series. In Table 3, the results of 16 cultures utilizing the new concentrations are summarized.

Initially we used only chloroquine and a control which yielded culture data as shown in Figure 2, but it was later realized that a reference culture was required. Since *P. falciparum* in Thailand is generally considered to be sensitive to quinine⁸, a culture containing 8 millimicromoles of quinine base per ml blood was added to the culture system. This simplified culture interpretation and resulted in the following system of grading the resistance of the parasites:

- (1) If both concentrations of chloroquine produce growth curves near the quinine growth curve the culture is rated sensitive (S) (Figure 3).
 - (2) If the 0.2 millimicromole chloroquine curve approximates the control curve and the 0.4 millimicromole chloroquine curve approximates the quinine curve, the culture is rated R+.
 - (3) If both concentrations approximate the control growth curve the culture is rated R++ (See Figure 4).
- In vivo* studies were possible in 50% of the malaria cases seen. Follow-up visits were requested for days 7, 14 and 21 after being admitted to the study program. In only a few cases was this schedule actually achieved.

A reproducibility study was performed by utilizing three identical wells for each harvest time and drug concentration. All wells were processed independently and counted to provide a 99% probability of 1% counting error.

* Carbon 14 Isoleucine. New England Nuclear Corp., NEC 278, Lot # 605-072 with Specific Activity 273 millicurie/millimole.

RESULTS: The in vitro growth multiplication rate of P. falciparum with C^{14} Isoleucine as a marker confirmed a minimum multiplication of five fold through one cycle of schizogony. This was determined by comparing the slopes of the growth curve before and after schizogony. This determination was facilitated by the fact that during schizogony no C^{14} Isoleucine is incorporated as reported earlier by Cohen et al for H^3 leucine incorporation^{9,10}. When maximum growth was desired, the media was changed after 48 hr. to remove harmful metabolic products from the system. The culture system designed for drug testing showed slightly lower multiplication rates (i.e. 2-3 fold) because the media could not be changed.

In all cultures containing quinine, a depressed growth curve was consistently evident as compared to the control, indicating the susceptibility of parasites to the antimalarial agent. In case of resistance, the growth curve approximated that of the control. This was also confirmed by morphological studies in three of these cultures (Table 4) of which one in vivo follow-up was possible, and in this case there was good correlation between in vivo, in vitro and morphological observations. It appeared that in the presence of antimalarial agents, susceptible parasites could not survive through schizogony, while resistant parasites escaped the antimalarial drug action and the penetration of merozoites into new red cells was evident.

The maximum variance observed in the reproducibility study was 10%, the normal variance was 3 to 5%. Counting efficiency was determined to be 58 to 64% using the channels ratio counting method.

In vivo follow-up studies were disappointing in that few patients returned for examination beyond day 7. The in vitro results compare favorably with in vivo parasitemias recorded on day 7, but less so for those few patients who returned for examination after that time. Patients Y-39 and Y-41 in Table 2 supported the in vitro findings of resistance with positive blood slides by day 21 but Y-44 did not bear out the prediction of susceptibility by becoming positive by day 14. Of those 5 patients shown in Table 3 as having had follow-up studies two were resistant both in culture and clinically, while three which produced susceptible cultures became positive on the 14th or 21st day. The possibility of reinfection could not be ruled out during this season of active transmission.

DISCUSSION: The culture system reported upon consistently produces successful culture growth over a sufficient period of time allow meaningful studies, in this case morphological and radiochemical observations. The number of culture failures has been less than 1% and were usually attributed to power failure or the administration of an antimalarial compound prior to patient study. The technic lends itself to the field because it does not require elaborate and sophisticated equipment during the initial culturing procedures nor must the technicians be as highly trained as with many in vivo culture systems. The cultures can be quenched at the field site and transported back to a base laboratory for channels ratio counting. Investigations underway indicate that cultures can originate from outside a country with subsequent acceptable laboratory results.

The selection of a site in which to perform the in vitro cultures is based on the availability of laboratory space, electricity and an available patient population with malarial. The centrifuge, refrigerator, incubator, ultraviolet chamber, plates and reagents are readily transported in one vehicle. Frequently in areas where malaria persists as a major health problem, electricity is absent. At one of the early field sites, electricity was provided by a SKW generator with emergency 300 W Honda generators for additional support, and successful cultures were obtained.

As was the case in this study, the available patients most usually comprise an agricultural population intent upon proceeding to their homes and farms and who are reluctant to return to the provincial medical facility unless they have actually relapsed or recrudesced. Thus, the validity of such an in vitro test is difficult to measure. This is particularly true during the rainy season when transmission is at its peak. To assure that a patient has not been reinfected is generally impossible. Too, the presence in most Thai villages of quack doctors who possess and dispense a wide assortment of substances, including chloroquine, may tend to confuse and distort the findings of patients who do return for further examination. It is possible that

apparent susceptibility of parasites in vivo as determined by blood examination two or three weeks after initial detection could be due to drug pressure. Unless the patient can be closely observed for the full 28 days prescribed by WHO⁸ there will generally be doubtful correlation between an in vivo study and in vitro data. It is tempting to use the field expedient suggested by WHO⁸ whereby assessment of resistance is based on the response of asexual parasitemia during the first week of treatment, with further observations providing evidence of recurrence of parasitemia which could only be presumptive if re infection cannot be ruled out. The series of patients referred to in Table 2 provide some correlation between clinical and culture results when the first week's response only is considered, with just 2 in vitro resistant cases being negative on day 7 (Y-39 and Y-41). When applied to the patients of Table 3, there are 5 instances of agreement and 4 where the findings in the patient on day 7 differ with the conclusions obtained from the cultures. In the latter even all were resistant by the in vitro technic which could have presumably resulted in the prescribing of a drug other than chloroquine.

The culture results substantiate the findings of Colwell¹¹ that P. falciparum in Thailand is mostly resistant to chloroquine, a fact which impairs the evaluation process for any in vitro test. Complete evaluation of the technique incorporating the radiolotope C-14 requires that a strain of malarials which is susceptible to chloroquine be investigated. If sufficient cases of sensitive P. falciparum can be found to establish confidence in the test procedure for non-resistant strains, an essential baseline could be described. The mean growth curve for susceptible strains would provide the necessary reference for comparison with the quinine growth curve, a control curve and the growth curve of the strain to be tested. To continue evaluation of this test and to provide the required characterization of the minimum response of a susceptible strain, investigations are currently underway at a research station outside of Thailand.

SUMMARY: A test for assessing the in vitro susceptibility of Plasmodium falciparum to chloroquine has been described. The culture system utilizes the protein incorporation of C¹⁴-isoleucine as a growth marker. Culture failure was low, reproducibility was good and there was a multiplication rate of 2:1 or better in 90% of the study cases. The culture results obtained by measuring the C¹⁴ incorporation provide an excellent correlation with morphological observations. In this preliminary report it was not possible to follow sufficient cases of clinical malaria to establish the necessary in vitro-in vivo correlation. Further studies are in progress with parasites less resistant to standard chloroquine chemotherapy.

Table 1.
Microtitre Plate Scheme

Type of Culture	Harvest Time			
	23 hrs	40 hrs	48 hrs	63 hrs
Microscopic Control (No C ¹⁴ -isoleucine)	0	0	0	0
C ¹⁴ Growth Control	0 (XX)	0 (XX)	0 (XX)	0 (XX)
0.2 millimicromole Chloroquine base/ml	0 (XX)	0 (XX)	0 (XX)	0 (XX)
0.4 millimicromole Chloroquine base/ml	0 (XX)	0 (XX)	0 (XX)	0 (XX)
8 millimicromole Quinine base/ml	0 (XX)	0 (XX)	0 (XX)	0 (XX)

Microtitre Plate Scheme. 0=Principal culture wells harvested to determine growth curve.
X=Culture wells harvested to assess reproducibility of culture procedure.

Table 2.
Comparison of in vitro and in vivo studies of 8 patients using chloroquine drug level 8 millimicromole/base/ml blood* in patients from Yala, Thailand.

Patient No.	<u>In vitro</u> Result	Parasitemia		
		Day 7	Day 14	Day 21
Y-36	R	+		
Y-39	R	-	+	+
Y-41	R	-	-	+
Y-44	S	-	+	
Y-45	R	+		
Y-46	R	+		
Y-47	R	+		
Y-48	R	+		

* Based on serum level at 12 hours after commencing standard oral therapeutic course of Chloroquine diphosphate.

Table 3.
Comparison of in vivo and in vitro studies of 16 patients
with Plasmodium falciparum from Yala, Thailand.

Patient No. Yala	<u>In vitro</u> Result	Parasitemia		
		Day 7	Day 14	Day 21
51	R	-	+	
52	PF*			
53	S	-	-	+
54	R			
55	R			
55 B	R+			
56	S			
56 B	R++			
57	R++			
58	R++	+		
59	S	-	+	
60	S	-	+	
61	R++	-	+	
62	R++	+		
63	R++	-		
64	R++	-		

NOTE: PF*=power failure.

Table 4.
Microscopic Evaluation of In vitro Culture Growth

Patient #	Time	Control				0.2 milli CQ/ml.				0.4 milli CQ/ml.				Quinine			
		R	M	S	Total	R	M	S	Total	R	M	S	Total	R	M	S	Total
Y 56 B	24	8	44	7	59	28	33	1	62	34	23	—	57	39	18	—	57
<u>In vitro</u> *	40	22	3	47	70	9	16	29	54	16	21	14	51	27	24	—	51
Resistant	47	44	19	53	116	37	26	35	98	32	22	27	81	26	30	—	56
Y 59 B	24	14	31	—	45	13	26	—	39	23	19	—	42	20	14	—	34
<u>In vitro</u> *	40	3	15	23	41	11	24	—	35	22	12	—	34	21	16	—	37
susceptible	47	14	11	30	55	9	22	8	39	20	9	—	29	16	22	—	38
Y 60	24	3	14	5	22	13	9	—	22	10	13	—	23	12	12	—	24
<u>In vitro</u> **	40	20	5	13	38	17	6	—	23	12	11	—	23	10	15	—	25
susceptible	47	48	6	9	63	22	9	7	28	15	9	1	25	12	19	—	31

R = Young Ring

M = Mature Trophozoite

* No clinical follow-up.

** Parasitemia on day 14

S = Schizont

Note: Counts are for 10,000 RBC.

AVERAGE SERUM CHLOROQUINE LEVELS OF THAI VOLUNTEERS
AFTER ORAL DOSAGE OF 1500 MG BASE

708

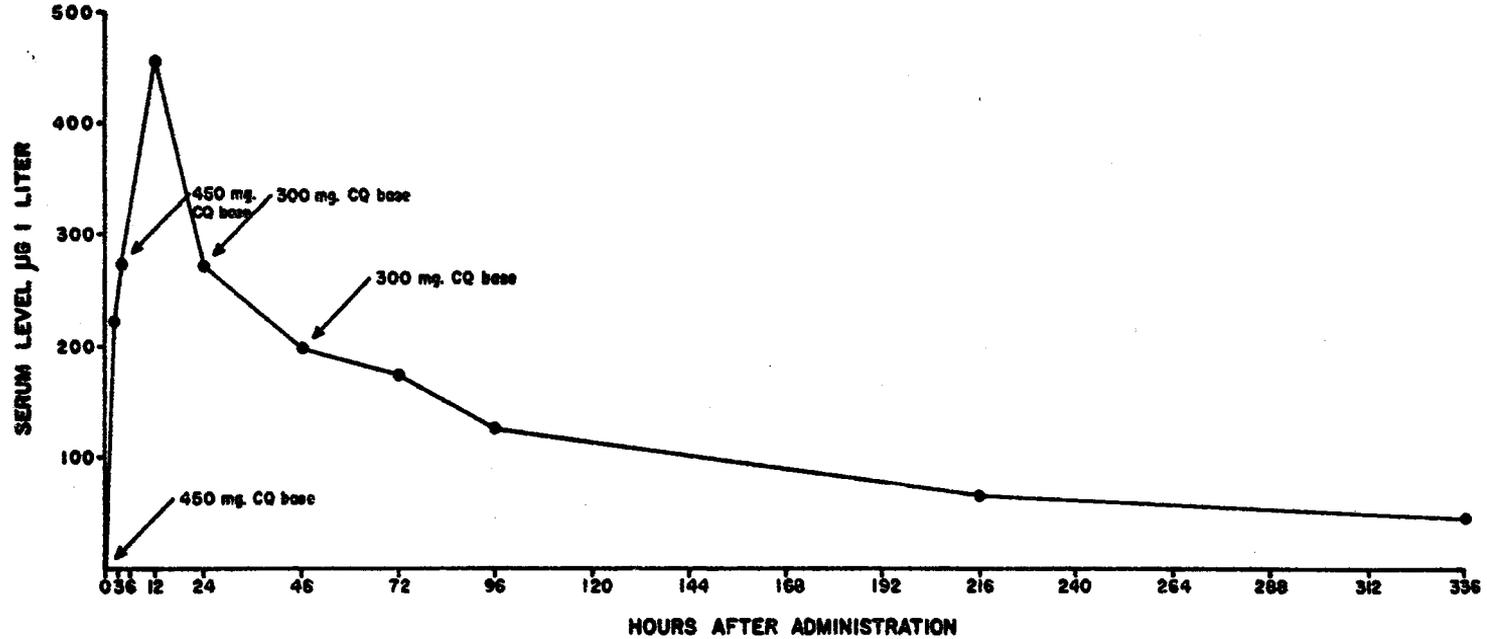


FIGURE 1

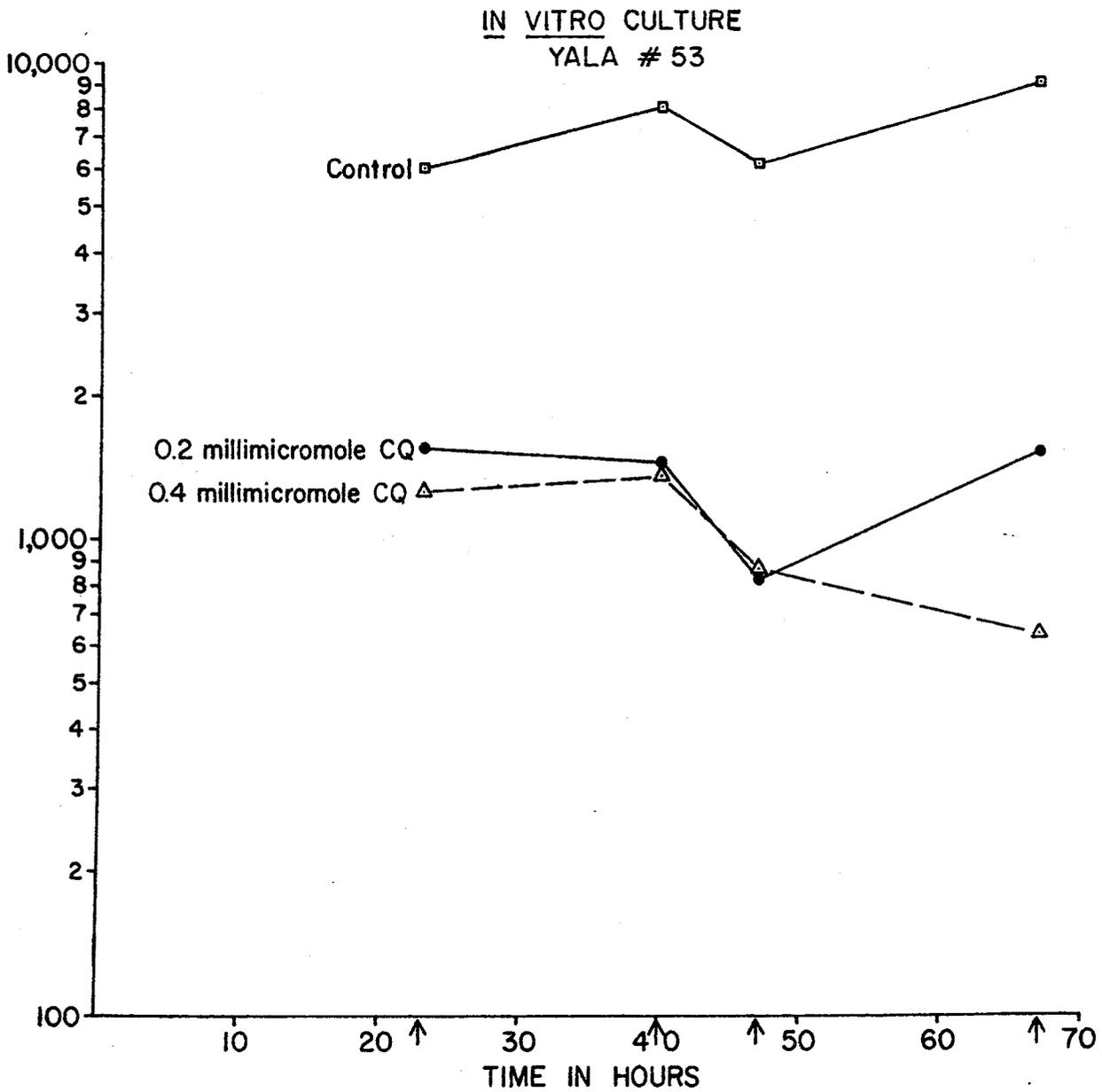


FIGURE 2.

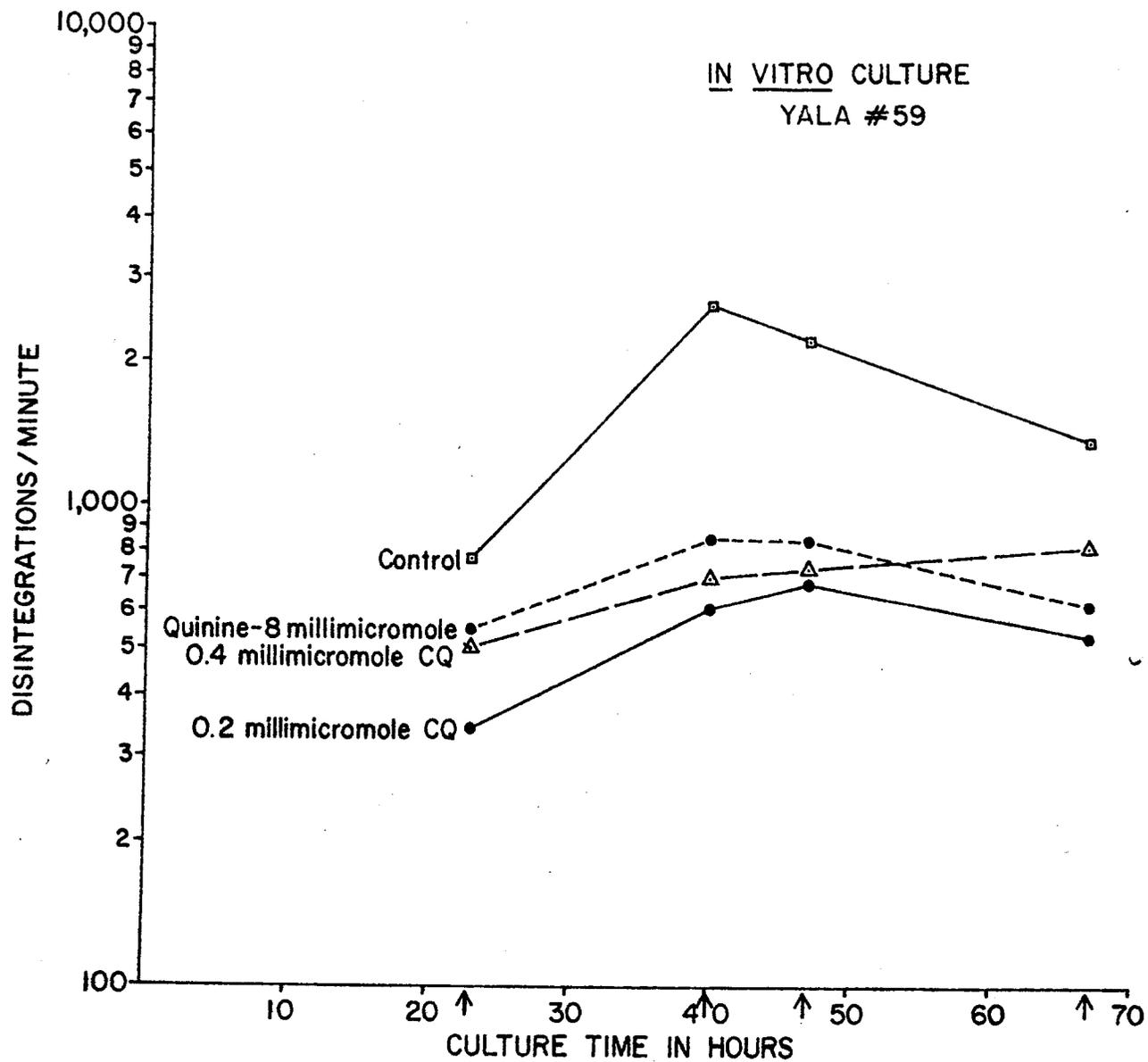


FIGURE 3.

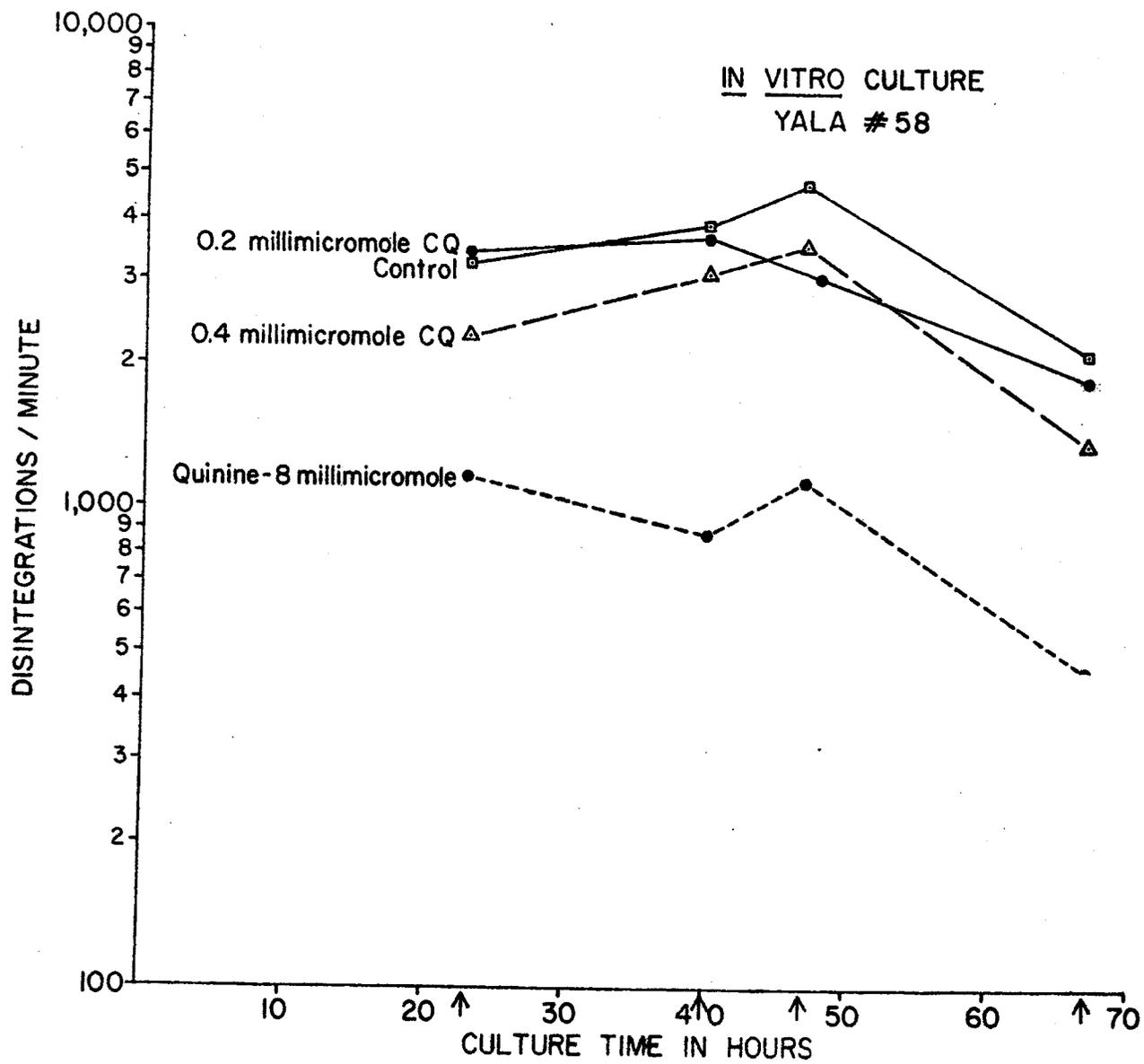


FIGURE 4.