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With 5 figures in the text

In vitro formation of ookinetes and functional maturity of *Plasmodium berghei* gametocytes

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SUMMARY

In vitro formation of *Plasmodium berghei* ookinetes was studied. Gametocytes produced *in vitro* were obtained from heart and tail blood of Swiss mice and from blood removed from mosquitoes directly after feeding on these mice. *In vitro* produced gametocytes were obtained from short-term cultures of the erythrocytic stages of *P. berghei*. Reproducible ookinete production was obtained in medium RPMI 1640, pH 7·8–8·0, using *in vivo* and *in vitro* produced gametocytes. The morphology of developmental stages of ookinetes and degenerate forms at the light microscope level is described. More ookinetes were produced in medium RPMI 1640 compared to MEM and ookinete yield – defined as the ratio between the number of *in vitro* produced ookinetes/10^5 erythrocytes and the number of exflagellations/10^5 erythrocytes in the infected blood – increased with lower erythrocyte densities in the cultures within the range of dilutions tested. A linear relationship existed between gametocytaemia and the number of ookinetes produced. The methods for *in vitro* ookinete formation and for estimating ookinete yields enabled us to study aspects of functional maturity of gametocytes independent of mosquitoes. The numbers of exflagellating gametocytes and *in vitro* ookinete yields in tail blood corresponded with those in heart blood and blood ingested by mosquitoes, suggesting a random distribution of functionally mature gametocytes within the vertebrate host.

INTRODUCTION

In malaria parasites gametogenesis, fertilization and ookinete development occur within the midgut of vector mosquitoes. These processes are influenced by factors present in the ingested blood (reviewed by Carter & Gwadz, 1980) and by mosquito factors. The latter may have stimulating (Nijhout, 1979) or inhibiting effects (Gass, 1977; Gass & Yeates, 1979).

Mosquito factors can be excluded by using techniques for *in vitro* fertilization and ookinete development. *In vitro* production of *Plasmodium berghei* ookinetes was reported by Weiss & Vanderberg (1977) and Chen, Seeley & Good (1977). The purpose of the present study was to obtain reproducible methods for *in vitro* ookinete formation using *in vivo* and *in vitro* produced *P. berghei* gametocytes and for estimating the number of ookinetes in relation to gametocyte numbers. These methods enabled us to study aspects of functional maturity of gametocytes (i.e. the capability to produce gametes and subsequent zygotes) independent of the mosquito.

In rodent malaria parasites the relation between functional maturity and morphology of gametocytes is poorly understood. In *P. yoelii*, differences in the morphology were

observed between microgametocytes in blood ingested by mosquitoes and microgametocytes in blood taken from the tail of infected mice. It was suggested that the infective gametocytes are distributed preferentially within the capillaries of the vertebrate host (Landau, Miltgen, Boulard, Chabaud & Baccam, 1979). We used our technique for *in vitro* ookinete formation to investigate the spatial distribution of functionally mature gametocytes of *P. berghei* within the vertebrate host.

MATERIALS AND METHODS

In vitro *ookinete formation*

For *in vitro* ookinete formation culture media RPMI 1640 (Gibco; 10·41 g/l) and MEM Earle's Salts (Gibco; 9·53 g/l) were used, without bicarbonate, both supplemented with inactivated foetal calf serum (10 % v/v) and Hepes (5·94 g/l) and adjusted to pH 7·8–8·0 with 1 M NaOH. MEM has successfully been used for culturing ookinetes by other workers (Weiss & Vanderberg, 1977). RPMI 1640 was chosen since it is suitable for culturing erythrocytic stages of *P. berghei* (Janse, Mons, Croon & van der Kaay, 1984). Infected erythrocytes, obtained as described below, were added to the media to give a suspension with a cell density of about 2×10^8 cells/ml. These suspensions were incubated at 20 ± 1 °C and 80–90 % humidity in 24-well culture plates (Costar, Cambridge, USA; 2 ml/well) or in 96-well microtitre plates (Nunc, Denmark; 0·2 ml/well).

Gametocytes from different blood sources

In vivo formed gametocytes were obtained from Swiss mice (20–25 g), 7 days after intraperitoneal infection with $0{\cdot}8–1{\cdot}2 \times 10^5$ *P. berghei* (ANKA-strain) infected erythrocytes. The ANKA-strain of *P. berghei* had been maintained by frequent transmission through *Anopheles atroparvus* (Van der Kaay & Boorsma, 1977). Only parasites derived from a second or third mechanical passage were used.

Heartblood (0·8 ml) was collected by cardiac puncture under ether anaesthesia and suspended in 5 ml of heparinized (2 i.u./ml) RPMI 1640 medium, pH 7·3 (Mons, Janse, Croon & van der Kaay, 1983). This suspension was washed (200 ***g***, 10 min) once with non-heparinized medium and the packed cells were resuspended in medium at pH 7·8–8·0.

Tail blood was collected in heparinized capillary tubes (Propper, Long Island City, N.Y.) and immediately suspended in medium at pH 7·8–8·0.

Blood ingested by mosquitoes: *Anopheles atroparvus* females (S-strain; Van der Kaay & Boorsma, 1977) were allowed to feed for a period of half an hour on a restrained mouse. Within 1 min after feeding, fully engorged mosquitoes were collected, midguts were dissected and the contents of the midguts of individual mosquitoes were mixed with medium at pH 7·8–8·0 and dispensed each into a separate well of a microtitre plate.

In vitro produced gametocytes were obtained from short-term *in vitro* cultures of the erythrocytic stages of *P. berghei* (ANKA-strain), established according to Janse, Mons, Croon & Van der Kaay (1983). Only once (20 h after start of the culture) fresh erythrocytes were added to the cultures. After 65 h the cultured cells were centrifuged at 200 ***g*** for 10 min to remove the medium. The packed cells were resuspended in RPMI 1640 at pH 7·8–8·0.

Parasite counts

After suspending infected erythrocytes in medium at pH 7·8–8·0 for 21–24 h, samples with an erythrocyte density of approximately 8×10^7 cells/ml were taken from the wells

and the number of ookinetes in 0·8–2·4 × 10^5 erythrocytes was counted in a Bürker haemocytometer. At a magnification of 400× mature ookinetes could easily be distinguished from other cells by their 'banana'-shape and by the presence of pigment granules. In each well we establish the ookinete yield. The ookinete yield is obtained by dividing the number of ookinetes/10^5 r.b.c. by the number of exflagellations/10^5 r.b.c. and multiplying this ratio by 100.

The number of exflagellations was counted before adding the infected erythrocytes to medium at pH 7·8–8·0. A sample of the infected erythrocytes was mixed with medium RPMI 1640 at pH 7·8–8·0. After 10 min at 21 °C this suspension, with a cell density of 0·8–1·2 × 10^8 cells/ml, was transferred to a Bürker haemocytometer. After the cells had settled, the number of exflagellations/0·8–1·2 × 10^5 erythrocytes was counted at a magnification of 400×.

Ookinete numbers are not presented in relation to the number of (macro)gametocytes but to the number of exflagellations since counting of (macro)gametocytes in smears not only is time-consuming but also is hampered by the non-random distribution of gametocytes. In Giemsa-stained smears of tail blood the percentage of infected erythrocytes was determined in 4–5 × 10^3 erythrocytes.

RESULTS

In vivo *produced gametocytes*

For *in vitro* ookinete formation blood was collected on day 7 post-infection (p.i.) from Swiss mice showing a rapid increase of infected erythrocytes from day 4 to day 7 p.i. The characteristic course of parasitaemia and the number of exflagellations/10^5 r.b.c. in tail blood from 10 of these mice during the first 9 days p.i. are shown in Fig. 1.

Maximum numbers of exflagellations in tail blood directly incubated in RPMI 1640 at pH 7·8–8·0 were reached on day 7 p.i. and ranged from 67 to 115/10^5 r.b.c. On days 6 and 7 p.i. the number of exflagellations was established in two blood samples taken from the tail within a short interval (10 min). Between these samples a maximum difference of 10% was observed (mean of 20 observations 5·5%). In mosquitoes fed on days 5–9 p.i. maximal oocyst production was found on days 6 and 7 (Fig. 1), indicating the presence of functionally mature gametocytes.

Standardization of the method for in vitro *ookinete formation*

In three experiments we investigated the influence of erythrocyte concentration on ookinete yield. Serial dilutions of the packed cells of infected heart blood were made with RPMI 1640 at pH 7·8–8·0, resulting in cell concentrations (v/v, packed cells/medium) as indicated in Fig. 2. Each dilution series was made with blood from a different mouse. In this case ookinete yields are expressed as percentages of the maximum ookinete yield observed in the relevant dilution series. The results show that ookinete yields increased with higher dilutions to a cell concentration of 2%. Therefore we used a cell concentration equivalent to an r.b.c. density of about 2 × 10^8 cells/ml in the experiments described below.

In 10 experiments tail blood was incubated in both RPMI 1640 and MEM. MEM yielded 15–67% less ookinetes compared to RPMI 1640 (Table 1). Table 1 shows that the variation in ookinete yield between different wells containing blood from one mouse was small (S.D. 5–13 in RPMI 1640) compared to the differences in ookinete yield between blood collected from different mice (S.D. 23·4 in RPMI 1640).

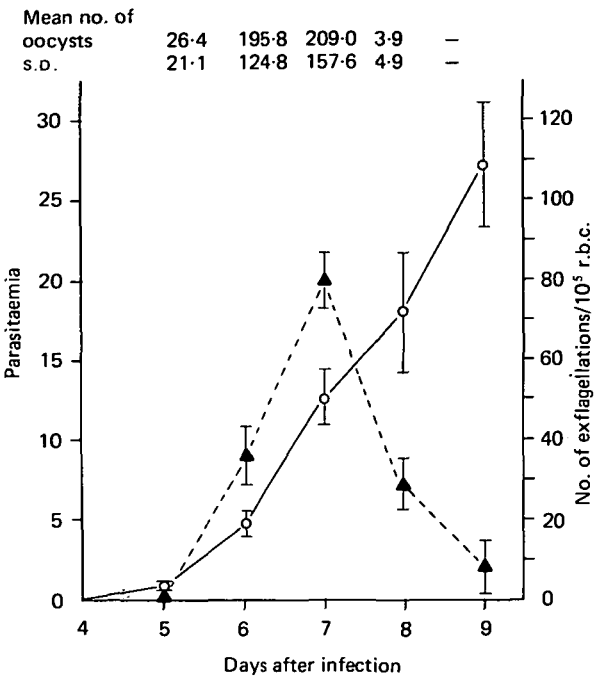

Fig. 1. *Plasmodium berghei* (ANKA-strain) infections in Swiss mice ($n = 10$). ○, Mean percentage of erythrocytes infected; ▲, mean number of exflagellations/10^5 r.b.c. Mice were infected intraperitoneally with $0{\cdot}8$–$1{\cdot}2 \times 10^5$ infected r.b.c. Oocyst numbers were established in *Anopheles atroparvus* mosquitoes 10 days after the infective bloodmeal. On each mouse 20–30 females, 7 days post-emergence, were fed on days 5–9 p.i.

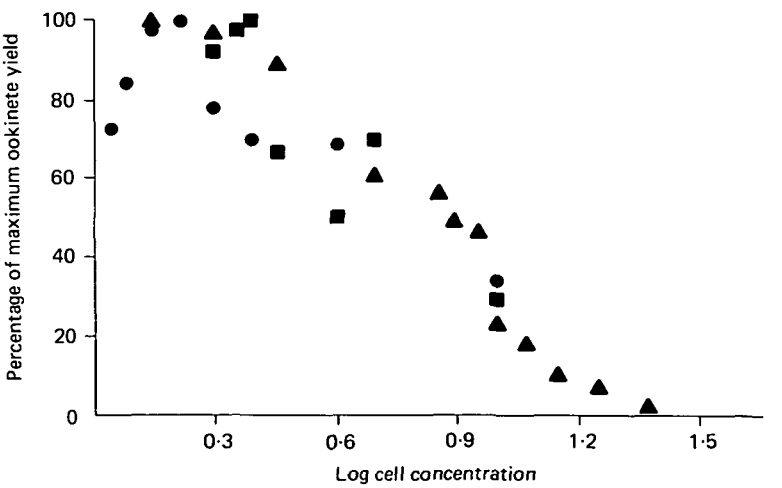

Fig. 2. Relationship between cell concentration (v/v, packed cells/medium) and ookinete yield. Serial dilutions of *Plasmodium berghei* infected heart blood from 1 Swiss mouse were made with medium RPMI 1640. Ookinete yield (mean of 5 wells) in each dilution is expressed as the percentage of the maximum ookinete yields observed in the 3 relevant dilution series. ●, ▲ and ■ represent 3 experiments. Maximum ookinete yields are: Exp. ●, 49; Exp. ▲, 50; Exp. ■, 39.

Table 1. *Number of exflagellations/*10^5 *r.b.c. and* in vitro *ookinete yields in tail blood of Swiss mice* 7 *days after i.p. infection with* 0·8–1·2 × 10^5 Plasmodium berghei *parasites*

(The number of exflagellations was established just before the tail blood was incubated in medium RPMI 1640 and in MEM Earle's salts. The ookinete yield is calculated by dividing the number of exflagellations/10^5 r.b.c. by the number of ookinetes/10^5 r.b.c. and multiplying this ratio by 100.)

Exp.	No. of exflagellations/ 10^5 r.b.c.	Ookinete yield (mean ± S.D.) in RPMI 1640	Ookinete yield (mean ± S.D.) in Earle's MEM
1	73	46 ± 12	17 ± 4
2	78	90 ± 13	48 ± 9
3	85	50 ± 8	24 ± 6
4	88	47 ± 9	30 ± 9
5	91	77 ± 10	55 ± 6
6	93	57 ± 5	19 ± 5
7	94	104 ± 9	67 ± 9
8	98	53 ± 10	32 ± 7
9	103	74 ± 13	25 ± 8
10	119	26 ± 9	22 ± 9
		$\bar{x}$ = 62·2	$\bar{x}$ = 33·9
		S.D. 23·4	S.D. 16·9

To establish a possible relationship between number of gametocytes/10^5 r.b.c. and ookinete yield we altered the number of gametocytes/10^5 r.b.c. artificially by serially diluting infected heart blood from one mouse with a suspension of uninfected erythrocytes before the heart blood was incubated in RPMI 1640. The resulting erythrocyte density was 1·5–2 × 10^8 cells/ml at all dilutions.

Results of three experiments (Fig. 3) show that ookinete yields remained constant irrespective of the gametocyte/ erythrocyte ratio.

Morphology of developing ookinetes

Ookinetes could be distinguished from other cells in the haemocytometer by their banana-shaped appearance and clearly visible pigment granules 21–24 h after start of the cultures. In 4 cultures we examined the morphology of developing parasites more thoroughly in Giemsa-stained smears since after culture for 21–24 h degenerated forms and young stages of ookinetes could be present but were unrecognizable in the haemocytometer. Smears were made every 3 h after suspending infected heart blood in RPMI 1640 and differential parasite counts were made on 200 parasites. Six different developmental stages of ookinetes (Fig. 4) were distinguished. Percentages of each stage are presented in Table 2.

We were not able to differentiate between unfertilized and fertilized macrogametes within 1 h of activation of the macrogametocytes. After 3 h, however, parasites were seen with a more condensed appearance and with a darker red staining, more extended nucleus (Stage I). These parasites seemed to develop a protrusion (Stage II). After 15 h Stage I parasites had disappeared but parasites, presumably unfertilized macrogametes, resembling the macrogametes seen during the first hour of culture, were still present. Stage III first appeared after 6 h. From 6 h onwards we constantly observed some parasites (maximally about 11 %) resembling Stage III in which, however, the nucleus

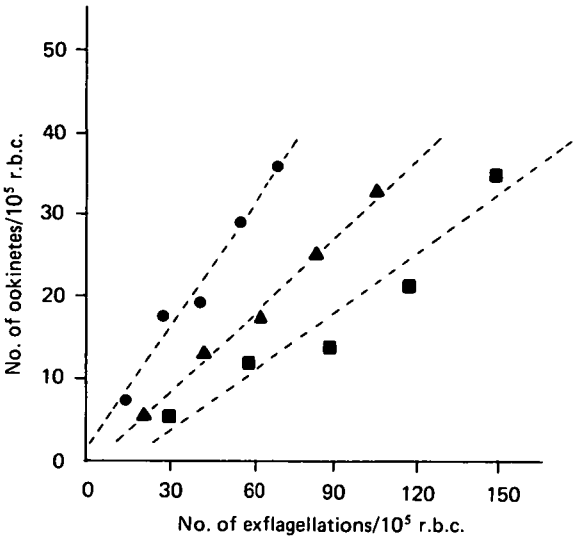

Fig. 3. Relationship between the number of exflagellations/10^5 r.b.c. and number of *in vitro* produced ookinetes/10^5 r.b.c. In each experiment *Plasmodium berghei* infected heart blood from 1 Swiss mouse was serially diluted with a suspension of uninfected r.b.c. before the blood was incubated in RPMI 1640. In all dilutions the final erythrocyte density was $1{\cdot}5–2 \times 10^8$ cells/ml. The ookinete number of each dilution is the mean of the ookinete number established in 5–10 wells. ●, ▲ and ■ represent 3 experiments. Exp. ●, $r = 0{\cdot}98$; $a = 0{\cdot}52$; $b = 0{\cdot}65$. Exp. ▲, $r = 0{\cdot}99$; $a = 0{\cdot}32$; $b = -1{\cdot}5$. Exp. ■, $r = 0{\cdot}96$; $a = 0{\cdot}96$; $b = -3{\cdot}5$.

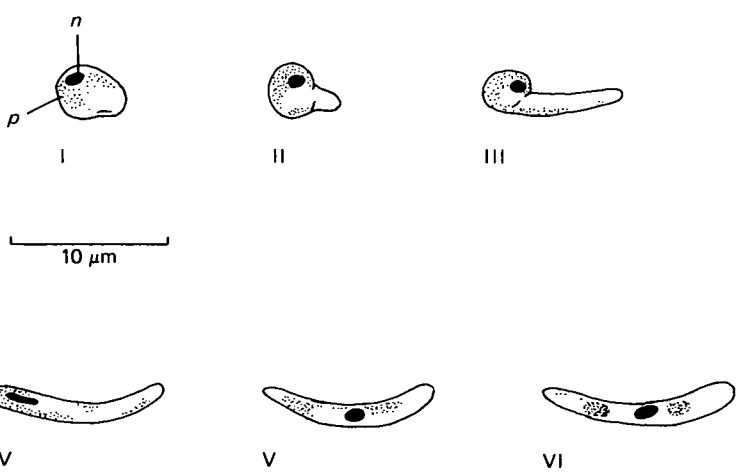

Fig. 4. A diagrammatic representation of the developmental stages of *in vitro* produced *Plasmodium berghei* ookinetes. The morphology was examined in Giemsa-stained smears at a magnification of 1000×. Stage I. Round or oval-shaped parasite. A more condensed appearance with a darker red staining and more extended nucleus (*n*) in comparison to macrogametes. Stage II. A little protrusion emerges from parasites resembling Stage I, filled with blue-staining cytoplasm without pigment granules. Stage III. First pigment granules (*p*) move into the enlarged protrusion. Nucleus still in the bulbous posterior end surrounded by pigment granules. Stage IV. First stage without round posterior end (banana-shaped). Nucleus migrates from the posterior end to the middle of the parasite. Stage V. This stage closely resembles Stage VI ookinete. Nucleus in the middle of the parasite. Dense pigment granules at one or both sides of the nucleus. Stage VI. Like Stage V but anterior end is enlarged and stains pink to red.

Table 2. *Percentages of six developmental stages (see Fig. 4) and abnormal forms of* Plasmodium berghei *ookinetes at different times in culture*

(The percentages are averages of 4 cultures. Differential parasite counts were made on 200 parasites in Giemsa-stained smears, prepared every 3 h after suspending infected heart blood of Swiss mice in medium RPMI 1640 at pH 7·8–8·0 at 20 °C. Forms classified as abnormal resemble Stage III parasites: the nucleus, however, was hard to distinguish from the pigment granules and there was generally a constriction between the bulbous posterior end and the protrusion.)

Hours after induction of gametogenesis	Developmental stage of ookinetes (% of total number)						
	I	II	III	IV	V	VI	Abnormal forms
3	100·0	—	—	—	—	—	—
6	62·8	22·3	9·4	—	—	—	5·5
9	21·6	33·1	36·5	2·6	—	—	6·2
12	5·7	15·2	54·1	7·5	7·7	—	9·8
15	—	6·3	24·0	30·5	26·3	2·1	10·8
18	—	—	5·4	13·2	50·4	21·2	9·8
21	—	—	—	3·9	32·2	53·7	10·2
24	—	—	—	—	20·4	68·6	11·0

was hard to distinguish from the dark pigment granules. In addition, these parasites showed a constriction between the bulbous posterior end and the light-blue protrusion, and often the bulbous posterior end and the protrusion of these parasites were found separated in smears, indicating the fragility of these forms. We consider them to be abnormal or degenerated parasites. Stage VI is considered to be the mature ookinete since it persisted for at least 12 h in culture. In the four cultures 10–17 % less ookinetes (all stages) were counted in smears at 24 h compared to 12 h, suggesting that some were lost during this period. From 21 h onwards only banana-shaped ookinetes and abnormal forms were seen.

Ookinete yields in different blood sources

Differential counts on 100–200 gametocytes in Giemsa-stained smears from tail blood of 10 mice indicated that 2–3 times more macro- than microgametocytes were present on days 6 and 7 p.i. (mean sex ratio ♀/♂ = 2·4). The percentage of erythrocytes infected with gametocytes on day 7 ranged between 0·2 and 0·5. Only gametocytes nearly or completely filling their host cell and sufficiently developed to allow sex differentiation were counted. Assuming that in the experiments shown in Table 1, too, 2·4 times more macrogametocytes than exflagellating microgametocytes were present, the percentage of female gametocytes transformed into ookinetes can be calculated from the number of exflagellations/10^5 r.b.c. at the time of withdrawal of the tail blood and the number of ookinetes/10^5 r.b.c. produced. This percentage ranged between 11 and 43 % in RPMI 1640.

In experiments carried out on days 4–9 p.i., the average number of exflagellations/10^5 r.b.c. in blood ingested by mosquitoes (n = 4) was compared to the average number in tail blood of the same mouse just before and just after feeding the mosquitoes (Fig. 5). In addition, mean ookinete yields in blood cultured from blood meals ingested by mosquitoes (n = 8) feeding on mice 7 days p.i. were compared to ookinete yields in tail

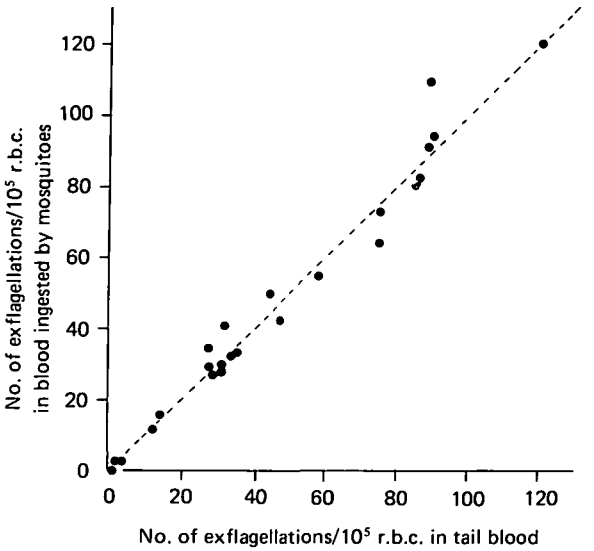

Fig. 5. Relationship between the number of exflagellations/10^5 r.b.c. in tail blood and in blood ingested by *Anopheles atroparvus* females. In each experiment the average number of exflagellations in blood ingested by mosquitoes ($n = 4$) is compared to the average number of exflagellations in tail blood of the *Plasmodium berghei* infected Swiss mouse on which the mosquitoes fed. Tail blood was collected just before and just after feeding the mosquitoes. The number of exflagellations was established/individual mosquito.

Table 3. In vitro *ookinete yields in different blood sources*

((*a*) Mean ookinete yields in blood cultured from the blood meal ingested by mosquitoes ($n = 8$) are compared to mean ookinete yields in tail blood (5 wells) of Swiss mice infected with *Plasmodium berghei*, collected just before feeding the mosquitoes. Ookinete yields were established/individual mosquito. (*b*) Mean ookinete yields in heart blood (5 wells) from Swiss mice infected with *P. berghei* are compared with mean ookinete yields in tail blood (5 wells), obtained just before the heart blood was collected.)

		Ookinete yield (mean ± S.D.)	
(*a*)	Exp.	Tail blood	Blood ingested by mosquitoes
	1	41 ± 13	38 ± 19
	2	26 ± 9	29 ± 9
	3	46 ± 15	54 ± 10
	4	105 ± 9	104 ± 9
	5	74 ± 13	74 ± 10
(*b*)	Exp.	Tail blood	Heart blood
	1	20 ± 3	19 ± 3
	2	46 ± 3	44 ± 8
	3	41 ± 4	49 ± 4
	4	21 ± 3	24 ± 6

blood collected just before the mosquitoes were fed on these mice (Table 3*a*). No significant differences were found between blood ingested by mosquitoes and tail blood of the same mouse, either in number of exflagellating microgametocytes or in ookinete yield. Since the number of exflagellations in blood ingested by mosquitoes corresponded to the number in tail blood, the ookinete yields in both blood sources were calculated by dividing the number of ookinetes by the number of exflagellations established in tail

blood just before the mosquitoes were fed. Assuming the same sex ratio as above (♀/♂ = 2·4) 11–44 % of female gametocytes in tailblood and 12–43 % of those in blood meals reached the mature ookinete stage *in vitro*.

In four experiments ookinete yields in heart and tail blood of the same mouse were compared. Heart blood was collected and treated in the same way as the tail blood, collected just before the heart puncture. The number of exflagellations differed maximally 16 % between the two blood sources and only small differences were observed in ookinete yield (Table 3*b*).

In *in vitro* cultures of erythrocytic stages of *P. berghei*, development of ringforms into mature microgametocytes took 26 h; these gametocytes retained the capability to produce gametes for a period of approximately 26 h (Mons, Janse, Boorsma & Van der Kaay, 1984). Exflagellation and *in vitro* ookinete formation were investigated using gametocytes from 4 short-term cultures. Each culture had been initiated with infected erythrocytes from a different rat and the percentage of infected erythrocytes had increased from 0·5–1 % to 6–8 % during the first 24 h of culture. The number of exflagellations/10^5 r.b.c. ranged from 0 to 4 at 40 h after the start of the cultures, and increased to 22–78 at 65 h, indicating that most of the microgametocytes originated from *in vitro* invaded merozoites. In the 4 individual cultures ookinete yields of 4, 7, 16 and 22 were found at 65 h.

DISCUSSION

Weiss & Vanderberg (1977), using essentially the same method for *in vitro* formation of ookinetes, estimated the percentage of macrogametocytes transformed into ookinetes at 1 %, which is far less than the percentages estimated in this study (8–44 %). They diluted infected blood from hamsters with a gametocytaemia of 3–10 % six times with MEM. In our hands MEM yielded 20–60 % less ookinetes than RPMI 1640. Moreover ookinete yields increased drastically with higher dilutions of the infected blood. Since gametogenesis will take place before the cells are settled in the wells, the release and mobility of microgametes may be less obstructed by other cells in the cultures with low cell concentrations. Within the mosquito midgut, however, fertilization and ookinete development occur in cell concentrations equal to or higher than that of blood. In other experiments we compared *in vitro* ookinete yields with those *in vivo*. We found 75–97 % less ookinetes/10^5 r.b.c. within the blood meal of mosquitoes 18 h after feeding, than in tail blood taken simultaneously from the mice on which the mosquitoes fed and incubated *in vitro* for the same period (unpublished results). This suggests that formation of ookinetes within the midgut is less efficient than in culture since mosquito blood meals, if taken and cultured *in vitro*, produced equal numbers of ookinetes to tail blood.

Apart from a possible lower chance of fertilization *in vivo*, the subsequent development of the ookinete within the midgut can be inhibited by mosquito factors. Gass (1977) and Gass & Yeates (1979) showed that *P. gallinaceum* ookinete development could be interrupted at the stage of a retort-shaped, immature form, by factors produced in the midgut of mosquitoes during blood digestion. In our cultures only small numbers of such retort-shaped forms (Stage III) failed to develop into banana-shaped stages.

Gao & Yuan (1982) described the *in vitro* development of ookinetes of the related parasite *P. yoelii*. They observed the first mature ookinetes after culture for 7 h. Several young stages were described having a constriction between the protrusion and a bulbous posterior end. In our cultures these forms were absent or present in low numbers and were obviously fragile. Therefore we classified them as abnormal or degenerated. In

several characteristics these forms resemble degenerated ookinetes of *P. gallinaceum* (Gass & Yeates, 1979).

In cultures with low erythrocyte concentrations a linear relationship existed between the number of gametocytes and the number of ookinetes when blood from an infected mouse was serially diluted with a suspension of uninfected erythrocytes. Thus the probability of fertilization did not decrease with decreasing gametocytaemia. Nevertheless, considerable differences in ookinete yields were found between blood from different mice. This implies that exflagellation is not a reliable indicator of the capability of gametocytes to produce ookinetes.

In vitro ookinete formation excludes mosquito influences on the process of fertilization and ookinete production. Since our methods for *in vitro* ookinete formation and for estimating the ookinete yield gave reproducible results, these are useful tools in the study of functional maturity of gametocytes produced either in the vertebrate host or in *in vitro* cultures of the erythrocytic stages. We were able to obtain ookinetes from *in vitro* produced gametocytes, showing their functional maturity. Reproducible *in vitro* ookinete formation using cultured gametocytes has not been reported earlier, although feeding of cultured gametocytes of *P. falciparum* to mosquitoes resulted in oocyst production (Sinden, 1983).

The relation between functional maturity and the morphology of *P. berghei* gametocytes is not clear. Landau *et al.* (1979) compared the morphology of *P. yoelii* microgametocytes in blood taken from the tail of mice and from mosquito midguts and showed that, on morphological criteria, the gametocyte population within the mosquito blood meal differs from that in tail blood.

In *P. berghei*, closely related to *P. yoelii*, we on the contrary found no evidence for a non-random distribution of functionally mature gametocytes. Both the number of exflagellating microgametocytes and the *in vitro* ookinete yield in tail blood corresponded with those in heart blood and in blood ingested by mosquitoes.

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