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Initial extracellular development *in vitro* of erythrocytic stages of malaria parasites (*Plasmodium falciparum*)

(extracellular culture/intracellular parasitism)

WILLIAM TRAGER, JAIME ZUNG, AND MARIKA TERSHAKOVEC

The Rockefeller University, New York, NY 10021-6399

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ABSTRACT **Merozoites of *Plasmodium falciparum* placed in culture medium with a 50% erythrocyte extract and supplemented with ATP and pyruvate differentiated extracellularly into early trophic forms. Erythrocyte extract prepared by sonication was found superior to extract prepared by freezing and thawing. Under the best conditions, up to 30% of the merozoites showed some development after 16 hr of incubation and ≈5% developed into larger forms, often ring-shaped and occasionally showing pigment. The small as well as the larger forms took up the fluorescent dye rhodamine 123. Under similar conditions, partial further development was also obtained of young rings freed from their host cells. Again, the sonicated erythrocyte extract gave better development than the frozen–thawed extract, and ATP with pyruvate had a marked favorable effect. These parasites had both a plasma membrane and a surrounding closely apposed parasitophorous membrane, whereas the forms derived by extracellular development of merozoites had only their plasma membrane. We conclude that initial development requires neither an intact erythrocyte nor a parasitophorous membrane.**

The propagation of a parasite *in vitro* apart from its intact host has always led to extensive progress in understanding host–parasite relationships. This is well illustrated with the growth in work on the biochemistry, immunology, and molecular biology of the human malaria parasite *Plasmodium falciparum* since its cultivation was first reported (1, 2). In these cultures, all the erythrocytic stages of the parasite, including male and female gametocytes as well as the asexual schizogonic cycle, develop within human erythrocytes maintained *in vitro*. Just as all other obligate intracellular parasitic protozoa that have been cultured, these parasites develop inside their living host cells. Can they be grown axenically? Why should a eukaryotic organism having its own protein-synthesizing machinery need to depend on a living host cell for its development, and what is the nature of this dependence? Might the host cell furnish triggers or signals for differentiation of the parasite as well as essential nutrients? These are basic questions in the biology of parasitism.

One approach toward answering these questions is to attempt to replace the living host cell with an environment in which the parasite can develop extracellularly. The value and importance of such extracellular cultivation of intracellular parasites has recently been emphasized by Moulder (3) in a thorough analytic review on intracellular parasitism. *P. falciparum* is especially well suited for such attempts, since the methods for its continuous culture make readily available all its erythrocytic stages and since so much is known about its host cell, the human erythrocyte.

There are now several lines of evidence that partial extracellular development of erythrocytic stages of malaria parasites is possible. In work with the bird malaria *Plasmodium lophurae*, trophozoites of this species, removed from their host duck erythrocytes with a hemolytic antiserum and complement, developed under appropriate conditions into schizonts in a 20-hr period of incubation at 40°C (4, 5). Essential to this development was a duck erythrocyte extract prepared in a nutrient medium of high potassium content and supplemented with certain cofactors. Most striking were the favorable effects of ATP with pyruvate and coenzyme A, all at physiological concentrations. This requirement for CoA (6) was also indicated by the lack of enzymes for biosynthesis of CoA in *P. lophurae* (7, 8). Viable extracellular *P. lophurae* were seen even after 3 days *in vitro* (9).

Nillni *et al.* (10) have found that the monkey parasite *Plasmodium knowlesi*, removed from host erythrocytes by nitrogen decompression, develops from forms with one to four nuclei into schizonts with five nuclei after 4 hr of incubation at 37°C in an "artificial intracellular medium." This medium has low sodium and high potassium, other ingredients as in Dulbecco's modified Eagle's medium, 10% heat-inactivated dialyzed calf serum, and a pH value of 6.7. Both the relatively low pH and the high potassium were considered important to this 4-hr period of development.

It needs emphasis that in the studies both with *P. lophurae* and with *P. knowlesi* the extracellular parasites were surrounded by the parasitophorous membrane closely apposed to their own plasma membrane. This was also the case in the very short term *in vitro* experiments on membrane potential of *Plasmodium chabaudi*, isolated from host rat erythrocytes by N_2 cavitation (11), and of *Plasmodium yoelii*, freed from mouse erythrocytes by saponin treatment (12, 13).

In contrast stands the surprising finding that was responsible for initiation of the present work. We observed that when schizonts of *P. falciparum* were incubated overnight in a medium of human erythrocyte extract supplemented with ATP and pyruvate, some of the merozoites emerging from them developed into small extracellular rings (14). Evidently, this differentiation was triggered not by the complex process of entry into an erythrocyte (15) but rather by the milieu in which the merozoite found itself. Furthermore, electron micrographs show that these early rings have no parasitophorous membrane, only their own single plasma membrane. Yet these forms take up rhodamine 123 (16), showing that they develop a transmembrane proton gradient across their single membrane and, incidentally, indicating their viability (13).

We present here further studies on this initial extracellular development of merozoites, with emphasis on the proportions of merozoites developing, on the role of ATP, and on improved results with a new type of erythrocyte extract.

MATERIALS AND METHODS

Preparation of Viable Merozoite Suspensions. Stock cultures of *P. falciparum* were maintained in flow vessels (5). We used two clones, A-2 of FCR-3/Gambia (17) and HB-3

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(18) of the Honduras I/CDC isolate. On a Wednesday the cultures were synchronized by sedimentation in PhysioGel (19), followed by a 3-hr period of invasion and finally by treatment with sorbitol (20). This left only the rings newly formed during the 3-hr period of invasion. These were returned to the flow vessel, which had meanwhile been thoroughly rinsed with culture medium. Two days later when mostly rings were present, the cultures were subcultured. Three days later when many late-stage parasites were present, the cultures were expanded into Petri dishes, each with 10 ml of 8% cell suspension and a starting parasitemia of 1%. Four flow vessels provided material for 48 dishes, and this was the usual number handled. These dishes were incubated at 37°C in candlejars (21) and received fresh medium on Tuesday afternoon and on Wednesday morning. At the latter time, sample slides were taken from eight of the dishes and from the four flow vessels. If the synchronization on the previous Wednesday had been timed so that invasion took place between 11 a.m. and 2 p.m., then many late schizonts and segmenting parasites would be present by about 3 p.m. a week later. All the material was centrifuged and made to 25% suspensions. These were layered in 20-ml amounts over 20 ml of 60% Percoll in culture medium held in 50-ml centrifuge tubes. Centrifugation for 20 min at 1500 × *g* yielded dark brown bands at the interface just above the Percoll. These bands had a parasitemia of ≈80%, consisting almost entirely of late schizonts. The material in each band was resuspended in medium with 15% serum (RP-15S) and centrifuged (8 min at 800 × *g*). The dark brown pellets were resuspended in RP-15S to give ≈3% suspensions, which were placed in 6-cm Petri dishes. The usual yield was four dishes, each with 5 ml. These were incubated in a candlejar at 37°C, with gentle swirling every 10 min (without removal from the candlejar), for 1.25 hr. The material in each dish was then placed in a centrifuge tube and centrifuged 10 min at 200 × *g*. This sedimented the schizonts, leaving a somewhat brownish cloudy supernatant with merozoites and pigment clumps. The supernatants were removed to fresh tubes and centrifuged 10 min at 1500 × *g*, sedimenting both merozoites and pigment clumps. Supernatant fluid was then removed to reduce the volume to about ¼–⅛, effecting a 4- to 8-fold concentration, and the merozoites were resuspended. It was found that merozoites concentrated in this way were as invasive as before treatment. The suspensions contained ≈200 × 10^6 merozoites per ml and were used to inoculate the experimental media and invasion controls (see below). All operations were done aseptically without the use of any antibiotic.

Preparation of Rings Freed from Their Host Erythrocytes. Schizonts from a synchronized culture were concentrated over 60% Percoll by the same methods used for the preparation of merozoites. They were washed once with RP-15S and the pellet, usually ≈0.1 ml, was mixed with 0.4 ml of 50% erythrocyte suspension and 40 ml of RP-15S. This suspension, with a hematocrit of 0.5%, was distributed in four Petri dishes of 10 ml each, and these were incubated overnight in a candlejar at 37°C. The next morning there was a 50% parasitemia with young rings. To remove most of the residual bodies and pigment, the cells were centrifuged and made to a 25% suspension. This was layered on a 40% Percoll layer on 60% Percoll and centrifuged 20 min at 1500 × *g*. The sedimented cells were washed once in RPS (standard culture medium with 10% serum) and then resuspended in 12× their volume of a complete erythrocyte extract medium with ATP and pyruvate (see below). To 4 ml of such a suspension was added 0.08 ml of guinea pig serum as a source of complement and 0.4 ml of rabbit antiserum from a rabbit immunized with washed pink erythrocyte ghosts of human type A+ erythrocytes and having a hemolytic titer of 1:5000. This mixture was incubated 30 min at 37°C with mixing by pipetting in and out at 15 min and again at 30 min. It was then centrifuged for 1 min at ≈80 × *g*. This sedimented agglutinated erythrocytes, including any parasites still surrounded by erythrocyte membrane, and left free parasites in suspension in the supernatant. The yield was 50% or better of the rings originally present.

The Culture Media. Two kinds of extracts from human erythrocytes have been used, one prepared by freezing and thawing, the other by sonication.

The preparation of the frozen–thawed extract, designated RCE, has been described (14). It is important to note that this extract was prepared from 1 vol of cells extracted in 2 vol of nutrient medium and centrifuged at high speed to remove the erythrocyte ghosts. It was equivalent to a 33% extract with respect to hemoglobin and other constituents of the erythrocytes. A 50% extract could not be prepared by the same method because the erythrocyte ghosts could not be removed. However, it was found that if the erythrocyte ghosts were agglutinated by the addition of a rabbit anti-erythrocyte antiserum, at 1.5 ml of serum to a mixture of 10 ml of frozen–thawed erythrocytes with 10 ml of the nutrient medium KE (see ref. 14), followed by incubation at 37°C for 15 min, a clear extract could be obtained after centrifugation for 2 hr in an angle head rotor at 40,000 × *g*. This extract was designated 50-RE. Both RCE and 50-RE were usually prepared 1 or 2 days in advance of an experiment and stored frozen at −70°C.

Sonicated extracts were prepared in the following way. The day before an experiment, whole blood (type A+), preserved in CPD-adenine and not outdated, was centrifuged at 500 × *g* for 10 min and the cells were washed three times in RP (complete culture medium of RPMI 1640 medium with hypoxanthine/Hepes/bicarbonate, but without serum) to remove leukocytes and plasma. The final pooled cells were resuspended in an equal volume of RPS and stored at ≈2°C. On the day of the experiment, this 50% suspension was centrifuged and the cells were washed once with an equal volume of KRPS. KRPS was a medium equivalent to RPS except that it was prepared with an RPMI 1640 medium powder in which all the NaCl was replaced by an equimolar amount of KCl. The cells were then made to a 50% suspension in KRPS.

Sonication was done by placing 5-ml portions of this 50% suspension in a 50-ml conical glass centrifuge tube. The tube was held in place in ice water in the cup horn attachment of a Heat Systems ultrasonic processor model W-380. The suspension was sonicated for 1.5 min at setting 3½ on the output control. The sonicates were kept in an ice bath and pooled in 12-ml amounts in 15-ml centrifuge tubes. They were centrifuged 10 min at 2000 × *g* to sediment any intact erythrocytes that might have been left. Usually there was no visible pellet. The upper 10 ml was removed from each tube. Note that this extract, designated KSon, contained hemoglobin and other erythrocyte constituents at ≈50% of their concentration in the erythrocyte. It also contained all of the erythrocyte membrane constituents fragmented to very small particles. It also differed from the frozen–thawed extracts in that the nutrient medium was not supplemented with a number of cofactors that had been included in the earlier work but found to be without effect. In some experiments, frozen–thawed extracts were prepared in KRP (equivalent to the KRPS used for the sonicate, but without serum) and the serum was added at 10% to the final stroma-free extract just before use. These are designated KF.

ATP and pyruvate were usually supplied together in a supplement, the preparation of which has been described (14).

ATP was measured by an enzymatic method using the no. 366-UV kit from Sigma. The oxidation of NADH was measured in a UV spectrophotometer at 340 nm.

Table 1. Better extracellular development of merozoites in a sonicate than in a frozen–thawed extract

Dish	Medium	Parasites per 2000 erythrocytes at 14 hr			
		S	L	L-P	Total
1	A	89	20	10	119
2	A	81	15	2	98
3	A +	70	17	7	94
4	GSH (8 mM)	84	21	11	116
5	A + GSH (8 mM)	76	6	10	92
6	+ CoA (0.15 mM)	76	14	8	98
7	B + GSH (8 mM)	33	10	2	45
8	+ CoA (0.15 mM)	33	5	5	43

The experiment was done in small Petri dishes holding 2 ml of medium and inoculated with 0.15 ml of a suspension with 125×10^6 merozoites of clone HB-3 per ml. These showed an invasion rate of 0.2%. Medium A was the sonicated extract KSon, plus 2 mM ATP and 5 mM pyruvate. Medium B was the frozen–thawed extract KF, plus 2 mM ATP and 5 mM pyruvate. GSH, glutathione. The counts were done without knowledge of the actual number of the slide. The total of ≈100 parasites per 2000 erythrocytes is derived from a 0.5-ml sample of a 2-ml culture inoculated with 0.15 ml of merozoite suspension, representing 0.25 or 0.035 ml. Since the initial count of merozoites was 250 per 1000 erythrocytes from a mixture of 0.05 ml of suspension with 5 μl of 50% erythrocytes, 0.035 ml would give 175 per 1000 erythrocytes. Since the best cultures had 50 extracellular forms per 1000 erythrocytes at 14 hr, ≈29% had developed. S, small; L, larger; L-P, larger with pigment.

Extracellular Cultures. During preparation of the merozoites or free rings, the media to be tested were completed so that they were ready a short time before the parasite suspension. Most experiments were done in 24-well Linbro plates, with 0.5 ml of medium in each well. For some experiments, small Petri dishes were used with 1.5 or 2 ml of medium per dish. After inoculation (usually at the rate of 0.1 ml of parasite suspension to 1 ml of medium), the Linbro plates and Petri dishes were held in a candlejar. All incubations were at 37°C.

Each experiment included controls to test the invasive ability of the merozoites. Usually two wells of a Linbro plate each received 0.05 ml of merozoite suspension mixed with 5 μl of 50% erythrocyte suspension and a droplet was removed for preparation of a thin film. The slide was stained with Giemsa stain and was used to count merozoites in relation to erythrocytes, from which the number of merozoites per ml was calculated. These wells were incubated in a candlejar at 37°C for 0.5 hr; each then received 0.5 ml of RPS and they were returned to the candlejar and 37°C. The next day, Giemsa-stained slides were prepared from these wells and were used to determine the number of rings per 1000 erythrocytes and, from this, the percentage of merozoites that invaded.

The experimental tests, usually after 14–18 hr of incubation, were prepared for evaluation in several ways. For stained preparations, to be used for counts, all or an aliquot of the thoroughly mixed material was centrifuged 15 min at $1500 \times g$. The supernatant was used for pH determination (and for other measurements, as of ATP). The residue was mixed rapidly with 5 μl of the same 50% erythrocyte suspension used on day 0 for the merozoite count. A thin Giemsa-stained film was prepared and used to count parasites, usually per 2000 erythrocytes. The number obtained could be compared directly to the initial number of merozoites per 1000 erythrocytes to give the percentage of merozoites that developed. To observe uptake of rhodamine 123, 5 μl of a rhodamine solution at 1 mg/ml was added for 0.5 ml of medium to give a rhodamine concentration of 10 μg/ml. After incubation under culture conditions for 30–60 min, the material was microcentrifuged 3 min, resuspended in culture medium, and again microcentrifuged 3 min. From the sediment so obtained, a wet mount was prepared and examined by epifluorescent microscopy at ×1250 using a BP 546 green interference combination exciter filter and an LP 590 red barrier filter with an FT 580 beam splitter.

RESULTS AND DISCUSSION

A consistent picture has emerged with regard to extracellular development of merozoites under the most favorable conditions. The number of merozoites inoculated correlates with the number of developing forms seen the next day on stained slides. The latter is from 10% up to 20% or even 30% of the number of merozoites, a very favorable proportion when we consider that infectivity of these same merozoite suspensions is around 1–3%. It is interesting that the proportion of larger extracellular forms is about 2–5% of the number of merozoites and, hence, of the same order as the rate of invasion. The number of merozoites also correlates with qualitative estimates of the number of organisms that fluoresce as a result of uptake of rhodamine 123. Whereas most of the fluorescent forms appear as small circular bodies, some are larger and often ring shaped. A few of these are gently motile and on occasion a tiny pigment grain can be seen. These correspond to the forms counted in stained films as "larger" and "with

Table 2. Better development of early rings freed from their host cells in a sonicate (KSon) than in a frozen–thawed extract (KF)

Well	Medium	Average counts per 1000 erythrocytes					% of total		
		S	L	L-P	Free troph.	Total	As L	As L-P	As free troph.
B: 1, 2	A (KF + ATP,	27	23	5	0	55			
5, 6	Py, and GSH)						24	15	4
C: 1, 2	B (KSon	43	22	13	1	79			
5, 6	+ ATP and Py)						27	13	2
D: 1, 2	C (same as B	42	22	13	2	79			
5, 6	+ GSH)						29	19	7

The lysate had 250×10^6 rings per ml. Each well of 0.5 ml received 0.1 ml of the lysate prepared in medium A. ATP was used at 2 mM, Py (pyruvate) was 5 mM, and GSH (glutathione) was 8 mM. After 1 day, wells 1 and 2 were used for stained films for counts. The counts shown are averages from the two slides for each medium. Since the initial count was 1000 parasites (none of them with visible pigment), at best ≈8% of the total survived. S, L, and L-P are defined in Table 1. troph., Trophozoites. Wells 5 and 6 received rhodamine 123 and were used for wet mounts and stained smears. The former showed many fluorescent parasites in all preparations. The smears were used to count parasites of different stages of development in a total of 200 in successive fields. The numbers give the average percent from the two slides.

Table 3. Effect of ATP and ADP with pyruvate (Py) or phospho*enol*pyruvate (PEP) on extracellular development of merozoites in 50-RE

Well	Addition to medium	[ATP], mM, at Start	[ATP], mM, at 1 day	Parasites per 2000 erythrocytes at 1 day S	L	L-P	Total
A: 1–5	Py (3.6 mM)	0.56	0.38	9	3	3	15
B: 1–5	Py (3.6 mM) ADP (3.2 mM)	0.52	0.80	13	6	2	21
C: 1–5	PEP (2 mM) ADP (3.2 mM)		1.0	13	3	3	19
D: 1–5	Py (3.6 mM) ATP (1.4 mM)	1.2	0.7	13	5	4	22

Each well holding 0.5 ml of medium was inoculated with 0.05 ml of a suspension of merozoites of clone A-2 of FCR-3 with an initial count of 55 per 1000 erythrocytes or $\approx 30 \times 10^6$ per ml. The invasion rate was 5%. At 1 day, three wells of each set were pooled in one centrifuge tube and the other two wells were pooled in a second centrifuge tube. All were centrifuged 15 min at $1500 \times g$ and each sediment was mixed with 5 μl of 50% erythrocyte suspension and used for a Giemsa-stained blood film. Numbers give the total counts for both slides for each medium divided by 5 to give the average per well. The highest totals of 22 per 2000 obtained from the initial 55 merozoites per 1000 erythrocytes represent a development of 20%. A sixth well of each set received rhodamine 123 at 10 μg per ml. Small bright fluorescent forms were numerous in all except A-6. In B-6, a clearly ring-shaped fluorescent form was noted. S, L, and L-P are defined in Table 1.

pigment." In cultures started with merozoites, such late rings have been the most advanced stages seen at 1 day. In cultures started with rings freed from their host cells, small numbers of evidently extracellular trophozoites have been seen the next day. Since no such forms could be found on the initial slides either before or after lysis, they must have developed in culture. Detailed experimental results to illustrate specific points are presented in the following sections.

Sonicated vs. Frozen–Thawed Erythrocyte Extracts. The 50% sonicates (KSon) in comparative experiments supported better development of both merozoites and lysed rings than the 33% frozen–thawed extracts (KF) (Tables 1 and 2). As shown in Table 1, development of merozoites in the sonicate was not affected by supplementation with glutathione or CoA and was twice as good as in the frozen–thawed extract. The pH of the sonicate remained close to 7.2, which is the internal pH of human erythrocytes (22, 23).

Similarly, rings freed from their host cells showed considerably better survival and development in the sonicate than in the frozen–thawed extract. It is true that the former represented a 50% extract, and the latter represented only a 33% extract. However, 50% frozen–thawed extracts (50-RE, see *Materials and Methods*) seemed also not to give as good results as 50% sonicates.

Effect of ATP and Pyruvate. The favorable effect of addition of ATP and sodium pyruvate was first seen with 33% frozen–thawed erythrocyte extract. It has also been obtained with the 50% frozen–thawed extract (50-RE) and with the 50% sonicates (KSon) (Tables 3 and 4, respectively). In Table 3, note that each number represents the average from two slides with material from five wells. ADP or ATP with either pyruvate or phospho*enol*pyruvate supported better development than pyruvate alone. Of interest is the relatively high level of ATP still present after 1 day and the formation of ATP from ADP, showing the presence of an ATP-generating system in the frozen–thawed extract with the stroma removed. The further addition to this medium of phosphocreatine with creatine kinase, or of pyruvate kinase, did not further improve the ATP levels at 1 day or the survival of the parasites (results not shown). In the KSon medium (Table 4), the ATP level fell markedly, but a favorable effect of an initially higher concentration was nevertheless evident. Other experiments showed that the fall in ATP occurred during the first 4 hr of incubation. We assume it results from the ATPase (24) present with the fragmented erythrocyte membrane in these sonicates. Also of interest in this experiment is the lower pH of inoculated as compared to uninoculated wells; this probably results from metabolic activity of the parasites, which were present in relatively high numbers.

The rapid decrease in ATP suggested a trial of supplementary ATP at 4-hr intervals. This did indeed have a favorable effect (Table 5). Whether the medium was without or with added CoA, the ATP pyruvate supplement at 4-hr intervals supported more developing forms: 23% of the merozoites showing some development with 6% becoming larger, as compared to 17% and 4% without the supplements.

ATP with pyruvate also favored the survival of early rings freed from their host erythrocytes. In an experiment done in the 50-RE medium, wells supplemented with ATP and pyruvate showed at 1 day twice as many total parasites and 4 times

Table 4. Early development of merozoites of HB-3 in a 50% sonicate (KSon) without and with supplemental ATP, pyruvate (Py), and glutathione (GSH), and the drop in ATP content

Well	Medium	pH on day 1 Inoculated	pH on day 1 Not inoculated	ATP, mM Day 0	ATP, mM Day 1	Developing forms per 2000 erythrocytes S	L	L-P	Average per 1000 erythrocytes Total	L + L-P
A: 1, 2	KSon	7.21	7.30	0.61	0.10	313	32	9	204	27
3, 4		7.20	7.30		0.11	395	39	27		
B: 1, 2	Medium A	7.14	7.23	2.13	0.13	412	78	19	215	37
3, 4	+ ATP (2.1 mM) and Py (5.4 mM)	7.11	7.22		0.10	299	42	9		
C: 1, 2	Medium B +	7.06	7.19	2.00	0.11	357	52	10	222	36
3, 4	GSH (8 mM)	7.06	7.19		0.09	386	68	14		

An uninoculated plate was prepared and incubated for 1 day under the same conditions as the plate that received in each well 0.05 ml of merozoite suspension with 330×10^6 merozoites per ml (counts on the two control wells were 672 and 662 merozoites per 1000 erythrocytes and 1 schizont per 10,000 erythrocytes for a mixture of 0.05 ml of merozoite suspension and 5 μl of 50% erythrocyte suspension). The invasion rate was 0.5% as seen in control wells on day 1. On day 1, the wells were pooled as two pairs for each medium and centrifuged, and supernatants were used for pH and ATP determination, and the residues were mixed with 5 μl of 50% erythrocytes and used for a stained slide. Hence, each slide represents material from two wells. The counts were done without knowledge of the slide number. The counts per 2000 erythrocytes for total parasites, and for larger forms plus those with pigment, from both slides were added and divided by 4 to give the averages per 1000 erythrocytes. S, L, and L-P are defined in Table 1. Note that ≈200 parasites per 1000 erythrocytes, or 100 for each well, developed out of the inoculum of 600 merozoites per 1000 erythrocytes, or ≈17%.

Table 5. Effect of supplementary ATP and pyruvate at 4-hr intervals on extracellular development of merozoites in KSon medium

Well	Medium	Developing forms per 2000 erythrocytes				
					Averages	
		S	L	L-P	L + L-P	Total
A: 3	A: KSon + ATP	99	27	6		
4	(2 mM) + pyruvate	102	23	16	32	129
5	(5 mM)	90	18	6		
B: 1	B: Medium A + 4-hr	140	30	14		
2	supplement	90	31	12	45	169
3		142	33	14		
C: 1	C: Medium A + CoA	63	13	11		
2	(0.1 mM)	121	26	7	30	118
3		79	22	12		
D: 1	D: Medium C + 4-hr	150	37	19		
2	supplement	106	26	11	41	162
3		100	27	9		

Each well received 0.05 ml of merozoite suspension with 200×10^6 merozoites per ml of clone A-2 of FCR-3. The invasion rate was 1%. Preparation of all wells was completed at 6 p.m. At 10 p.m., 2 a.m., and 6 a.m., each of the wells from B and D received 5 μl of a solution with dipotassium ATP (138 mM) and sodium pyruvate (364 mM), thus supplying ATP at 1.4 mM and pyruvate at 3.6 mM. At 10 a.m. of the following day, slides for counts were prepared. The counts were done without knowledge of the slide number. A fourth well for each of the four groups was provided at this time with rhodamine 123 and it was examined for rhodamine fluorescence 1 hr later. In A-6 and C-4, many fluorescent ring forms, including some fairly large ones, were seen. In B-4 and D-4, the picture was similar except that more larger forms were noted. S, L, and L-P are defined in Table 1.

as many larger forms and forms with pigment as did the unsupplemented wells.

Role of the Host Erythrocyte and the Parasitophorous Vacuole Membrane. The fact that merozoites of *P. falciparum* can differentiate extracellularly into early trophic forms and begin feeding shows that neither an intact erythrocyte, nor the complex process of entry (15), nor a parasitophorous membrane is required, at least at the start. Certainly the ATP of the erythrocyte, normally 1.4–2 mM, plays an important role both in triggering the initial differentiation and later in maintaining further development, as shown for both *P. lophurae* (5) and in the present work for *P. falciparum* in experiments with parasites freed by lysis of their host cells. Such parasites do have the parasitophorous membrane. It is relevant in this connection that an influx of ATP by means of an ATP/ADP translocator has been shown for *P. falciparum* freed from host cells by either immune lysis or N_2 cavitation and having their parasitophorous membrane (25). An adenylate translocator had been earlier demonstrated by Kanaani and Ginsburg (26) but was interpreted to function in the reverse direction, supplying ATP from the parasite to the host cell. In either case, it remains to be determined, and will be of much interest to know, whether the translocator is in both the parasitophorous vacuole membrane and the parasite's plasma membrane or only in the latter.

Similarly, in studies on membrane potential (11, 13) using tetraphenylphosphonium or rhodamine 123, the free parasites were surrounded by a parasitophorous membrane and the relative roles of this membrane and the parasite plasma membrane remained undetermined. The present findings with active uptake of rhodamine 123 by parasites that have developed extracellularly show clearly the existence of a potential across the parasite plasma membrane itself. Further ultrastructural studies of these forms will be reported separately.

Constituents of the erythrocyte cytoplasm as supplied in the concentrated extracts are essential. These of course supply hemoglobin. They might also furnish other high molecular weight factors. The somewhat better development observed in sonicated erythrocyte extracts than in frozen–thawed extracts was contrary to expectation. In frozen–thawed extracts, failure to remove the stroma (present as intact ghost membranes) had a deleterious effect in experiments with both *P. lophurae* and *P. falciparum* (14). In the sonicates, the stroma is completely fragmented and could not be removed by centrifugation at $40,000 \times g$. Furthermore, in the sonicates ATP fell rapidly to lower levels than in the frozen–thawed extracts. It might be that freezing and thawing damages some essential factor that survives sonication. Perhaps more likely is the possibility that erythrocyte membrane constituents are somehow required. These speculations are open to experimental trial.

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