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The evolution of the new permeability pathways in *Plasmodium falciparum*—infected erythrocytes—a kinetic analysis

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Abstract

Malaria parasites demonstrably increase the permeability of the membrane of the erythrocyte in which they develop and propagate. New permeability pathways (NPPs) generated by parasite activity and identified in the erythrocyte membrane are held responsible for these changes. Here, we present a novel analysis of hemolysis curves of infected cells in iso-osmotic solutions of solutes that penetrate selectively into infected cells, as a function of parasite development. The analysis yields three parameters: the $t_{1/2}$ of lysis (reciprocally related to permeability), the maximal lysis, and a parameter that expresses the variation of the cell population. Different developmental stages of the parasite were obtained either by sampling synchronized cultures with time or by the fractionation of asynchronous cultures on a Percoll–sorbitol density gradient. While the results confirm previous reports on the stage-dependent evolution of NPPs, they also reveal that the evolution of NPPs is not synchronous: NPPs evolve differentially throughout the ring stage and only at the mid-trophozoite stage they are fully deployed in the majority of the infected cells, but not in all. This leads to desynchronization in the culture and to less than the maximal possible rate of multiplication.
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Index Descriptors and Abbreviations: *Plasmodium falciparum*; New permeability pathways; Iso-osmotic lysis; Kinetic analysis

1. Introduction

The pathogenic stage of the malaria parasite is confined to the erythrocytes of the infected host. Life within this compartment requires vast changes in the permeability properties of the membrane of the infected erythrocyte to allow for the extensive traffic of substrates and waste products needed for the growth and propagation of the parasite. Indeed, the membrane of the infected erythrocyte becomes highly permeable to various types of solutes including anions and cations (Kirk, 2001). The new permeability pathways (NPPs) display anion channel properties that discriminate between non-charged solutes according to their size. The origin of the NPPs is presently the subject of an animated debate, where some maintain that they are generated by a parasite-encoded protein (Alkhalil et al., 2004), while others favor the modulation of endogenous transporters of the erythrocyte (see for a recent review (Huber et al., 2005). Both electrophysiological data and an analysis of transport data (Ginsburg and Stein, 2004), suggest that there may be more than one type of channel. While most of the experimental effort in the realm of NPPs is devoted to their biophysical analysis and to the identification of their molecular origin, little is known about the dynamics of their generation during the life cycle of the parasite. In this investigation we address this issue using the semi-quantitative test of permeability, the iso-osmotic lysis technique (Ginsburg et al., 1983).

2. Materials and methods

2.1. Parasite cultivation and synchronization

Parasite strains FCR3, W2, and NF54 were cultivated as previously described (Shalmiev and Ginsburg, 1993) with

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the following modifications: the plasma concentration was 8% (v/v) and 50 μM hypoxanthine were added. Cultures were synchronized by two consecutive sorbitol treatments (Lambros and Vanderberg, 1979) so as to get a stage window of 6–10 h out of the 48 h of the cycle. Cultures were started at the ring stage and samples were taken 3–6 h apart for lysis measurements as described below.

2.2. Fractionation of parasite stages by Percoll–sorbitol gradient

Non-synchronous cultures were used. Percoll–sorbitol gradients were prepared as described before (Kutner et al., 1985). The method is based on the differential permeability of erythrocytes harboring different stages of parasite development and the density of infected cells. Cells with high permeability (e.g., trophozoites and schizonts) take up sorbitol to a greater extent than cells that have lower permeability (e.g., ring stage), and swell accordingly following the osmotic entry of water. Thus, cells harboring increasingly mature stages are less dense because of their increased permeability and decreased intrinsic density (Krugliak et al., 2002), and therefore settle in the less dense layers of the Percoll gradient. For optimization of the fractionation, the following alterations of the original protocol (Kutner et al., 1985) were made: for each culture, two discontinuous gradients were prepared, one for the enhanced separation of trophozoite stages consisting of (from top to bottom) 50, 55, 60, 65, 70, and 90% Percoll, and for the separation of ring stages, 60, 70, 75, 80, 85, and 90% Percoll. Packed cultured cells were diluted 1:1 in RPMI 1640 supplemented with 300 mM sorbitol. For the first gradient they were incubated for 5 min at RT, whereas for the second gradient cells were incubated for 20 min at 37 °C. Cells were then overlayed on the gradient and, after 20 min centrifugation at 12,500*g* at RT, the gradient layers were removed and washed (5 min, 500*g* at 37 °C) three times, first in wash medium (growth medium without plasma; WM) that contains 100 mM sucrose, then with WM + 50 mM sucrose, then with WM + 25 mM sucrose, and finally with WM. These washes are essential for allowing the sorbitol that has penetrated the cells to exit gradually; otherwise the osmotic gradient would have drawn water into the cells and lysed them. After 1 h in WM at 37 °C to allow for the remaining traces of sorbitol to exit the cells, thin blood smears were prepared, stained with Giemsa and parasitemias of the different stages were determined (usually each layer contained only one stage), and cells were counted in a hemacytometer. Suspensions were adjusted to the 1% hematocrit and taken for lysis measurements.

2.3. Measurement of lysis

Cells were spun for 10 s in an Eppendorf centrifuge at 15,000*g* and resuspended in an equal volume of 300 mM solute (sorbitol or arabitol, or xylitol) buffered with 10 mM Tris–HCl at pH 7.4. Samples were taken at different time intervals, centrifuged, and the supernatant was taken for measurement of optical density at 415 nm, close to the peak absorption of hemoglobin. For total hemoglobin content, a sample of the initial suspension was lysed in 19 volumes of ice-cold water and the optical density in the supernatant was measured. For suspensions that were less than 100% parasitemia, the total content represents both infected and uninfected erythrocytes. The % lysis of each sample was calculated by dividing its OD by that of the total. When all infected cells are lysed, the % lysis should be equal to the % parasitemia. However, as hemoglobin is digested during parasite maturation, % lysis thus calculated is expected to be lower at the more mature stages.

2.4. Data analysis

The % lysis values at different times were fitted by nonlinear regression using SigmaPlot® equation for a sigmoid dependence of y on x. $y = \frac{a}{1+e^{-(\frac{x-x_0}{b})}}$ where y is the % lysis, a is the maximal lysis, x is the sampling time, x_0 is essentially the $t_{1/2}$ of lysis, and b represents the variability of cells in the population. Were all lysing cells in the suspension identical, the lysis curve would look like a step function. This sigmoid shape reflects the variability in cell volume and in permeability. That volume variability exists in normal erythrocytes has been documented before (Ponder, 1948). Intraerythrocytic parasites develop at different paces as evidenced by the fact that it is very difficult to keep a synchronized culture synchronous for more that one cell cycle.

3. Results

3.1. Lysis of infected cells fractionated on a Percoll–sorbitol gradient

A typical set of lysis curves resulting from the lysis of infected cells retrieved from different layers of the Percoll–sorbitol gradient, in iso-osmotic sorbitol, is shown in Fig. 1. The derived half-times ($t_{1/2}$) were plotted against the Percoll concentration in the layer from which the cells were taken (Fig. 2): the age of parasites increases with decreasing Percoll concentrations.

Demonstrably, $t_{1/2}$ drops with parasite maturation. Since the reciprocal of this value is directly proportional to the permeability coefficient, the data indicate more than a doubling of the permeability to sorbitol with parasite maturation. Greater increases (~4-fold) were seen previously when permeability was measured by radioisotopes (Ginsburg et al., 1986).

It is expected that when infected cells containing the new permeability pathways are exposed to isotonic solutions, they will eventually lyse, and release all their hemoglobin. The released hemoglobin eventually serves for the estimation of lysis (see Section 2). This is essentially seen in Fig. 3 where the maximal lysis parallels parasitemia. However, when the first value is divided by the second, instead

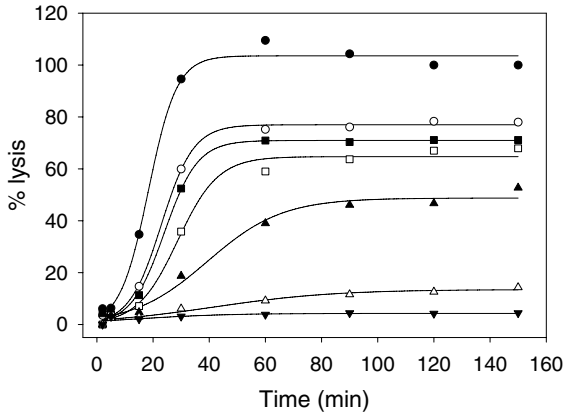

Fig. 1. Cells retrieved from a Percoll–sorbitol gradient were incubated at RT in a solution of 300 mM sorbitol + 10 mM Tris–HCl. The lysis at different time intervals was calculated. The continuous lines represent the best fit to a sigmoid function. Percoll concentration in layer from which cells were retrieved (%): 55% (●); 60% (○); 65% (■); 70% (□); 75% (▲); 80% (△); 85% (▼).

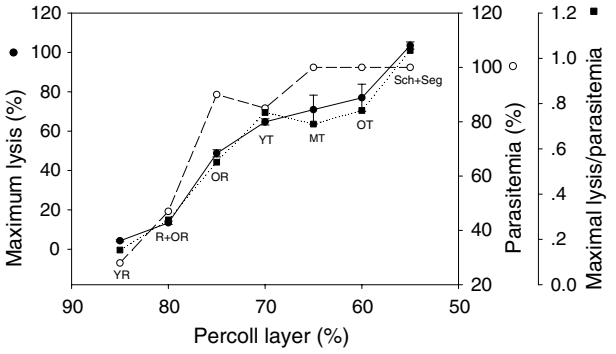

Fig. 3. The derived maximal lysis values from the data shown in Fig. 1 were plotted against Percoll concentration from which the infected cells were derived. The parasitemia in each layer is also shown (notice different in scale of right and left ordinates). The maximal lysis values were divided by the parasitemia in each layer and plotted. These data reflect the fraction of cells that were actually lysed. Parasite stages are indicated next to each data point. YR, young rings; R, rings; OR, old rings; YT, young trophozoites; MT, mid-term trophozoites; OT, old trophozoites; Sch, schizonts; Seg, segmentors.

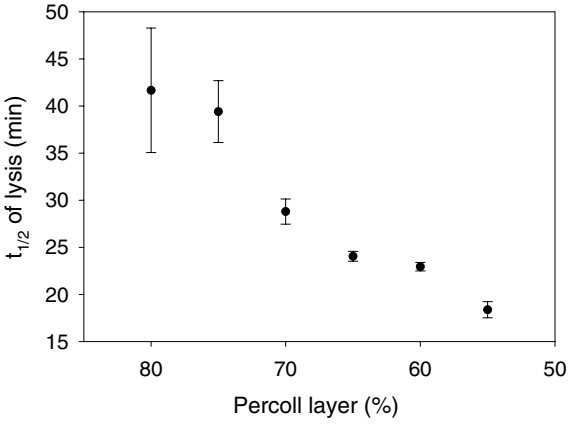

Fig. 2. The half times of lysis derived from the data of Fig. 1 by non-linear regression fitting to the sigmoid function, were plotted against the concentration of Percoll in the gradient layer from which they were derived–parasite maturation goes from left to right.

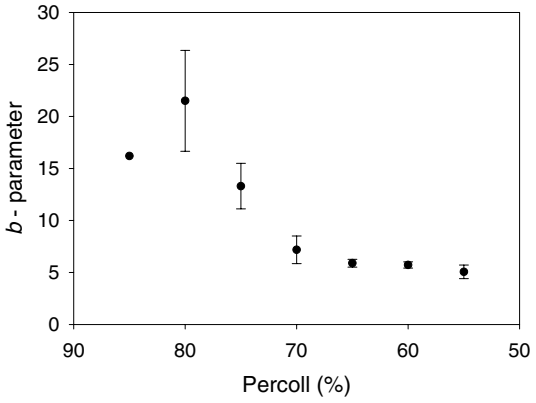

Fig. 4. The *b* parameter retrieved from the non-linear regression of the % lysis vs. time shown in Fig. 1 using the sigmoidal equation is plotted against the layer of Percoll. This parameter reflects the variability of sensitivity to lysis in the cell population.

of getting throughout values close to one, the ratio increases with parasite maturation (see filled squares in Fig. 3). The possible reasons for such a behavior will be dealt with in the Discussion.

Plotting the *b* parameter in the different Percoll layers, indicates that it decreases with parasite maturation (Fig. 4).

The same experiments were performed with different strains and different solutes and essentially the same features were observed. However, since the permeability depends on the size of the solute (Ginsburg et al., 1983), the $t_{1/2}$ values for the smaller solutes xylitol and arabitol are necessarily shorter than those for sorbitol (Table 1).

A close inspection of the table indicates that the NPP induced by W2 are significantly less permeable than those of the other strains suggesting that either the number of channels/cell is smaller or, less likely, that the aperture of the channel in W2 are narrower. The average increase in permeability (decrease in $t_{1/2}$) for all solutes and strains is 2.54 ± 0.67, meaning that the number of NPPs increases by not more than 2.5-fold with parasite maturation.

Table 1
Derived $t_{1/2}$ of lysis for various solutes and parasite strains

Strain	Solute	Highest $t_{1/2}$	Lowest $t_{1/2}$	Ratio of $t_{1/2}$
NF54	Xylitol	6.93	2.52	2.75
W2	Arabitol	40.40	12.30	3.28
FCR3	Arabitol	7.73	4.58	1.69
FCR3	Sorbitol	38.61	15.98	2.42

3.2. Testing the permeability of NPP during the development cycle of the parasite

Could the less than full lysis in Percoll-separated infected cells, result from the manipulation of the cells prior to the lysis experiment (loading with sorbitol and its

subsequent wash-out, contact with Percoll)? To verify this possibility, we have used tightly synchronized cultures and followed their development, taking samples every 4–6 h and measuring their hemolysis.

Typical results involving the FCR3 strain and the solute arabitol are shown in Figs. 5 and 6. In Fig. 5 the maximal lysis values are plotted as a function of time after infection. It is clear that even when NPPs start to be expressed the lysis is not complete. Only around 20–25 h post-infection when trophozoites predominate, the lysis reach ~90% of the possible maximum (the % maximal lysis divided by the parasitemia). Later in the cycle, the maximal lysis drops as would be expected from the demonstrable digestion of the host cell's hemoglobin (Krugliak et al., 2002). This reduction of maximal lysis however, may be also due to the failure of younger stages seen in the Giemsa-stained thin blood smears to lyse altogether because they did not develop NPPs (synchronicity was seen to decline with time in the cycle; data not shown). The $t_{1/2}$ of lysis reaches its maximum at the mid-trophozoite stage (~22 h) and remains the same thereafter (Fig. 6). Similar results were obtained for this and other strains with sorbitol (data not shown). The variability in osmotic susceptibility as reflected by the parameter *b*, declines with parasite development (Fig. 7).

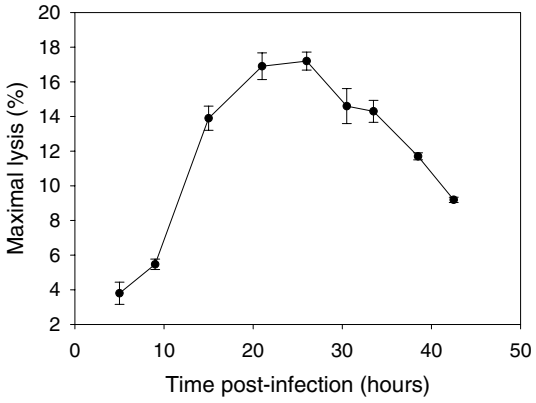

Fig. 5. A culture of the FCR3 strain was synchronized by two consecutive sorbitol treatments and cultured at 1% hematocrit and 18% parasitemia, starting from the early ring stage. Samples were taken at 4–6 h intervals and tested for their lysis kinetics as induced by their exposure to iso-osmotic arabitol. Lysis curves were fitted to the sigmoid model and the derived maximal lysis was plotted against the time in the parasite cell cycle.

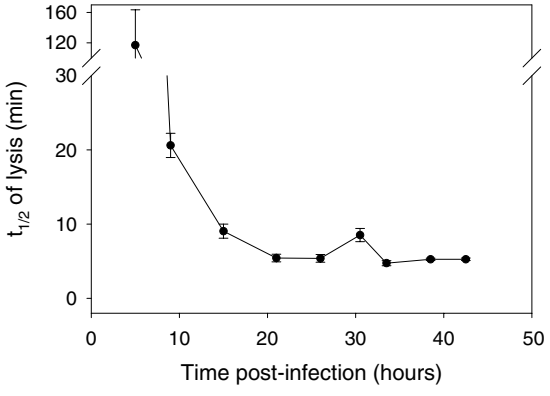

Fig. 6. The half time of lysis was derived by non-linear regression from the lysis kinetics of samples taken at 4–6 h intervals in the cell cycle and plotted against the time of sampling.

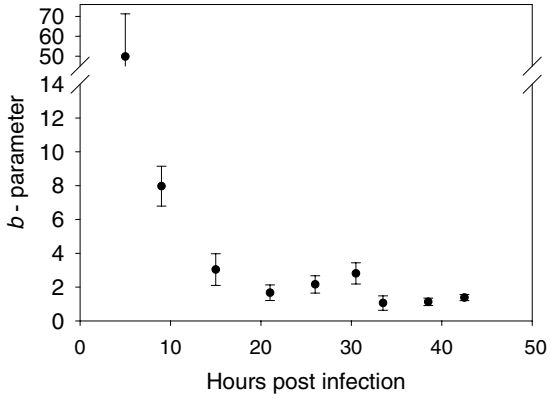

Fig. 7. The *b* parameter retrieved from the non-linear regression of the % lysis vs. time using the sigmoidal equation is plotted against the time in the parasite cycle post-infection. This parameter reflects the variability of sensitivity to lysis in the cell population.

4. Discussion

The appearance of increased permeability in the membrane of the plasmodium-infected erythrocyte to otherwise impermeable solutes, was recorded more than two decades ago (Ginsburg et al., 1983, 1985). Much of the work done since has relied on the seminal technique used then, the lysis in iso-osmotic solutions of the tested permeant. The method has been criticized in principle for being (1) restricted to polar solutes that are not metabolized, (2) for requiring the use of non-physiological conditions, including the possibility that the permeation route may be saturated at concentrations much lower than the 300 mM needed for istotonicity (Kirk, 2001). Non-metabolized polar solutes were used in the present investigation. In a recent analysis (Ginsburg and Stein, 2004), we have shown that permeability coefficients derived from lysis are very similar to those calculated from influxes measured by radiotracers at more physiological conditions. Thus, after having satisfied both criteria we justifiably make use of the great advantage of the iso-osmotic lysis technique: (1) it measures specifically the permeability of the membrane of the infected cell without perturbing the integrity of the host-parasite arrangement; (2) it is simple and cheap to use and allows high throughput screening of different solutes and potential inhibitors of NPPs (Kang et al., 2005).

Although many efforts have been invested in the characterization of NPPs (see (Huber et al., 2005 for a recent review), much less has been discovered with regard to their evolution during the development and maturation of the parasite. An attempt to bridge this gap is made in this work.

Three parameters were retrieved from the kinetic analysis of the course of lysis of infected cells in iso-osmotic solutions of penetrating solutes: the maximal extent of lysis, $t_{1/2}$ of lysis that is inversely proportional to the permeability, and the b parameter which reflects the variability among the cells in the population. As expected and shown before, the permeability of the host cell membrane increases with parasite maturation as evidenced by the decrease in $t_{1/2}$ (Figs. 2 and 6) (Ginsburg et al., 1986; Staines et al., 2001). We note that in the latter report, infected cells were enriched from cultures using Percoll cushions, indicating that contact with the constituents of Percoll that were used in this investigation do not affect the properties of the NPPs. In the present investigation the permeability (corresponding to the reciprocal of $t_{1/2}$; see (Ginsburg and Stein, 2004) for the exact relationship) was determined either by following the evolution of parasite development in synchronized cultures, or by separating the various stages by the functional expression of the NPPs on Percoll–sorbitol density gradients. The similarity of the results (compare Figs. 2 and 6) implies that both techniques for the stage-dependent evolution of NPPs are valid.

The outstanding result of the present investigation that emerges from the maximal lysis values, is the partial lysis of infected cells. That is, not all cells lyse as would be expected from an even and full development of NPPs in all infected cells: even if the numbers of NPPs are small at the early stages of parasite development, infected cells should eventually lyse, albeit at prolonged times of incubation. The uneven evolution of NPPs is more pronounced at the early stages of parasite development. As the parasites mature lysis is seen to be more uniform and complete. Although, incomplete lysis has been observed in the past (e.g., Figs. 1 and 4 of Ref. (Ginsburg et al., 1983) and Figs. 1 and 2 of Ref. (Ginsburg et al., 1985), it has neither been analyzed in detail nor interpreted in any way.

Before proceeding to analyze the present results, it is important to ascertain that incomplete lysis is not an experimental artifact. It has been known for a long while that erythrocytes lysed in hypotonic solutions reseal spontaneously (Bodemann and Passow, 1972). Resealing may be affected by ionic strength; it is known to be affected by temperature—the higher it is, the faster is the resealing (Bodemann and Passow, 1972). If resealing following lysis is fast, some of the hemoglobin could be retained in the lysed cells and an apparent less-than-full-lysis will be observed. We have tested the effect of temperature in the range of 15–37 °C and although the rate of lysis changed as expected, maximal lysis remained the same (data not shown). Furthermore, since the membrane of erythrocytes infected with ring stage parasite are the least modified by parasite activity, their spontaneous resealing should be similar to that of uninfected cells shown to be less than 17% at room temperature(Bodemann and Passow, 1972). Moreover, the hematocrit would be expected to affect the apparent maximal lysis: the higher it is, more hemoglobin is expected to be trapped in the lysed cells during their spontaneous resealing, yielding lower values of maximal lysis. This was not observed within the range of 0.1–5% hematocrit (data not presented). Incomplete lysis cannot be attributed to the presence of Percoll since it was also observed in synchronized cultures that have never seen this agent (Fig. 5). In view of these critical tests, the less-than-full-lysis cannot be related to changes known to occur in the structure and function of the infected erythrocyte with parasite maturation (Sherman, 1985) and should be considered as a true phenomenon.

All this said, the maximal lysis values must relate to the cell population present in the sample. Although, it is expected that if all infected cells that contain NPPs will eventually lyse, this does not happen even when the incubation time is prolonged to 3 h. In all cases and with both experimental protocols, only a small fraction of the young developmental stages do lyse. We interpret this result to mean that not all infected cells develop NPPs at the same time. With time in the parasite life cycle, NPPs gradually appear in most (and sometimes in all) infected cells, as evidenced by the fact that the fraction of cells that lyse increases with parasite maturation (Fig. 3). The Percoll fractionation technique yields layers containing less than 100% parasitemia, most notably at the higher Percoll concentrations that contain erythrocytes infected with young parasite stages. We have shown before that the lower than 100% parasitemia at these Percoll concentrations is due to the presence of lighter erythrocytes in the normal blood (Krugliak et al., 2002). If all infected cells retrieved from these layers do lyse, the % lysis should be equal to the % parasitemia. However, the ratio of maximal lysis to parasitemia reveals that this is not so (Fig. 3) indicating that a large proportion of young infected cells are devoid of NPPs.

The shape of the lysis curves (Fig. 1) indicate that the population of infected cells is rather variable, displaying normal distribution as far as lysis is concerned (Fig. 1). In the Percoll–sorbitol gradient technique, such variability is sometimes seen by microscopic inspection showing different stages in the same Percoll layer. However, the derived parameter b which represents the cell population's variance, was not found to reflect the observed morphological variability (Fig. 4), probably because the separation of cells is based on their transport function. Thus, morphologically-distinct stages may have the same numbers of NPPs, attesting again to their differential evolution during parasite maturation. Both in Percoll-enriched infected cells and in synchronized cultures, the b parameter declines with parasite maturation (Figs. 4 and 7) indicating a decrease in the variability of the osmotic susceptibility. Since NPPs are needed for normal parasite development (Kirk, 2001), the growth of parasites in those cells that do not develop NPPs is retarded. Thus, this differential development of NPPs results in desynchronization of the parasites in the culture or even in their death. We prefer the latter alternative since it could be the reason why the rate of parasite multiplication in culture is always lower than the number of merozoites per segmentor.

The reason for the differential development is not known. However, desynchronization also occurs in vivo (Boyd and Kitchen, 1937; Cheng et al., 1997). In a theoretical analysis (Hoshen et al., 2000) it was argued that desynchronization could be a result of the stochastic fluctuations in the individual periodicity of the parasite stage-cycle that are genetically determined. But the molecular mechanism underlying these stochastic fluctuations is not known. The differential development of NPPs, if it is not due to the less than optimal culture conditions, could be a plausible candidate for these fluctuations

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