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STUDY OF SOME PARAMETERS AFFECTING THE *IN VITRO* CULTIVATION OF *PLASMODIUM FALCIPARUM* WITHIN *SAÏMIRI SCIUREUS* RED BLOOD CELLS

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The in vitro *growth and multiplication of the erythrocytic stages of* Plasmodium falciparum *within* Saimiri sciureus *(squirrel monkey) red blood cells have been studied. Various parameters, such as the origin of the red blood cells and serum supplement, nature of the buffer, influence of the final pH of the medium, role of proteose peptone and glucose addition, were investigated.*

The selection of the best culture conditions led to the obtention of a reproducible in vitro *growth of two parasite cycles in* Saïmiri *erythrocytes, which is an useful achievement for* in vitro *studies.*

Our failure to establish a continuous culture line for longer than 19 days, could be explained by a dramatic increasing of osmotic fragility of the Saïmiri *red blood cells related to their small size.*

Key words: *Plasmodium falciparum – Saïmiri sciureus – in vitro* culture – *Saïmiri* erythrocytes

The continuous culture of *Plasmodium falciparum* was initiated by Trager and Jensen (Trager & Jensen, 1976) and rapidly revealed to be an important acquisition for malaria research. This basic technique has been used for numerous studies on morphology, biochemistry and nutritional requirements of the parasite, its relationship with the host-cell and the action of drugs. It also has allowed the exploration of the immunological defense mechanisms (Perrin et al., 1981) by detection of inhibitory activity of primate immune sera (Campbell et al., 1979; Green et al., 1981; Reese & Motyl, 1979). But in absence of *P. falciparum* culture in monkey red blood cells, the latter studies were restricted to a heterologous system.

In order to solve this problem and to improve the experimental model *P. falciparum*/ *Saïmiri sciureus*, we started assays of *P. falciparum* cultures using *Saïmiri* red blood cells.

We present here our initial results concerning growth of two strains of *P. falciparum.*

MATERIAL AND METHODS

Parasites – The Uganda Palo Alto (FUP) and Indochina I (Indo I) strains of *P. falciparum*, previously adapted to the squirrel monkey *Saïmiri sciureus* (Gysin & Fandeur, 1983), have been used throughout this work. These strains were initially provided respectively by R.J.M. Wilson (London, U.K.) and M. Hommel (Liverpool, U.K.).

Saïmiri **monkeys** – Squirrel monkeys, *Saïmiri sciureus* karyotype 14-7, originating from Guyana, were maintained at the "Institut Pasteur de la Guyane Française". Both intact and splenectomized monkeys were used as red blood cell (RBC) and serum donors. In an experiment, erythrocytes were taken twice from the same female: firstly just prior to the splenectomy and then ten days later.

Uninfected red blood cells – Blood, from intact and splenectomized *Saïmiri* monkeys or from a human O^+ donor, was collected by venipuncture into heparinized vacutainers (Becton Dickinson) and then centrifuged for 10 min at 800g.

The plasma and buffy coat were removed and the uninfected red blood cells (URBC) resuspended in RPMI (Gibco) and then centrifuged again. The cells were washed twice in the same way and then resuspended in an equal volume of RPMI supplemented with 10% foetal bovine serum (Flow) (FBS). They were stored in this medium, at +4°C for no longer than a month.

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Infected red blood cells – Infected red blood cells (IRBC) used to initiate cultures were obtained from highly infected (25%) splenectomized *Saïmiri*. They were collected and prepared as described above.

Sera – FBS, one *Saïmiri* monkey serum and two human sera were used after heat inactivation at 56°C for 30 min. The two human sera (A^+ and AB^+) were selected in terms of their low agglutinin titers to *Saïmiri* RBC (1:8 and 1:16 respectively). The human A^+ serum was absorbed with pooled washed *Saïmiri* erythrocytes to remove hemagglutinins.

Culture media – The culture medium was derived from that originally described by Trager and Jensen (Trager & Jensen, 1976). A few modifications were made concerning the buffer and the supplementing sera used: in some experiments *Saïmiri* monkey serum or FBS and glycylglycin or TES buffers were respectively substituted for human serum and HEPES buffer.* All the different media were prepared from a stock solution of RPMI, thereafter called RPMI stock (10.4 g RPMI 1640, 2.1 g $NaHCO_3$, and 10 mg gentamicine dissolved in 1 liter). The culture medium was buffered at a final pH of 6.9, 7.0, or 7.2 with either HEPES (50 mM, 30 mM, 3 mM); glycylglycin (150 mM, 80 mM, 20 mM) or TES (30 mM, 20 mM, 2 mM).

The final medium were occasionally supplemented with 0.2% W/V glucose or/and 0.15% W/V proteose peptone (Difco).

Culture procedure – A 50% erythrocyte suspension in RPMI was mixed with enough IRBC to give an initial parasitemia ranging from 0.15 to 1%. The mixture was further diluted with the different kinds of medium to give a 10% red cell suspension.

The cultures were set up either in small petri-dishes (3.5 cm diameter) or in 16 mm wells in a 24 well plate (Linbro).

For cultures in petri-dishes, each dish received 1.5 ml volume and the incubation was carried out at 37°C under a continuous flow of a humidified gas mixture containing 2% O_2, 3% CO_2, and 95% N_2.

For cultures in 24 well plates, each well received 0.5 ml volume and the plates were set up at 37°C in a single candle-jar (Jensen & Trager, 1977).

In all experiments, duplicates or triplicates were made for each category. The parasite growth was evaluated daily on Giemsa stained thin blood films.

Culture medium was removed every 24 hours and replaced by fresh medium.

RESULTS

In a first series of experiments, we tested the 96 hour growth in *Saïmiri* RBC of parasites derived from monkeys infected with the Palo Alto strain of *P. falciparum*. The cultures were carried out in 24 well plates. The following parameters were studied: origin of the supplementing serum (*Saïmiri* serum, A^+ or AB^+ human sera), buffer used (TES, HEPES, glycylglycin), influence of the pH of the medium, role of glucose and proteose peptone addition. All the results are shown in Fig. 1.

As reported previously with the *Aotus* monkey (Campbell et al., 1979), normal *Saïmiri* serum did not support the growth of parasites as well as normal human serum. Multiplication rates observed in wells containing human serum (Fig. 1 B/C) were higher than those obtained with *Saïmiri* serum (Fig. 1 A). The addition of serum in the culture medium was restricted in the case of *Saïmiri* serum to a 10% supplementation, higher concentrations resulted in poorer growth.

No significant differences were obtained when varying the buffer or the pH of the medium. The only exception took place in the wells of experiment B, showing that medium without glucose addition and buffered with HEPES gave multiplication rates inferior to those obtained with TES or glycylglycin buffered medium, and that all media buffered at a final pH of 7.2 led to poorer growth than that observed with a final pH of 6.9.

In all cases, the addition of 0.2% W/V glucose was beneficial to parasitic growth, except on one occasion (Fig. 1 C) when 0.15% W/V proteose peptone was advantageously substituted for a portion of the supplementing human serum.

In a second series of experiments, we selected the best culture conditions obtained from the short term culture assays reported above. Using RPMI culture medium buffered with 30 mM

**Abbreviations used in this paper:* GLG, glycylglycin; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid; IRBC, infected red blood cells; RBC, red blood cells; S^+, intact *Saimiri*; S^-, splenectomized *Saimiri*; TES, N-tris (Hydroxymethyl)-2-aminoethane Sulfonic Acid; URBC, uninfected red blood cells.

HEPES (pH 7.0) and supplemented with 0.20% W/V glucose, 0.15% W/V proteose peptone and 10% A^+ human serum, we succeeded in maintaining the Palo Alto *P. falciparum* strain in *Saïmiri* RBC during a 19 day continuous culture, 4 passages, total dilution of erythrocytes: 1:1.800 (Table I).

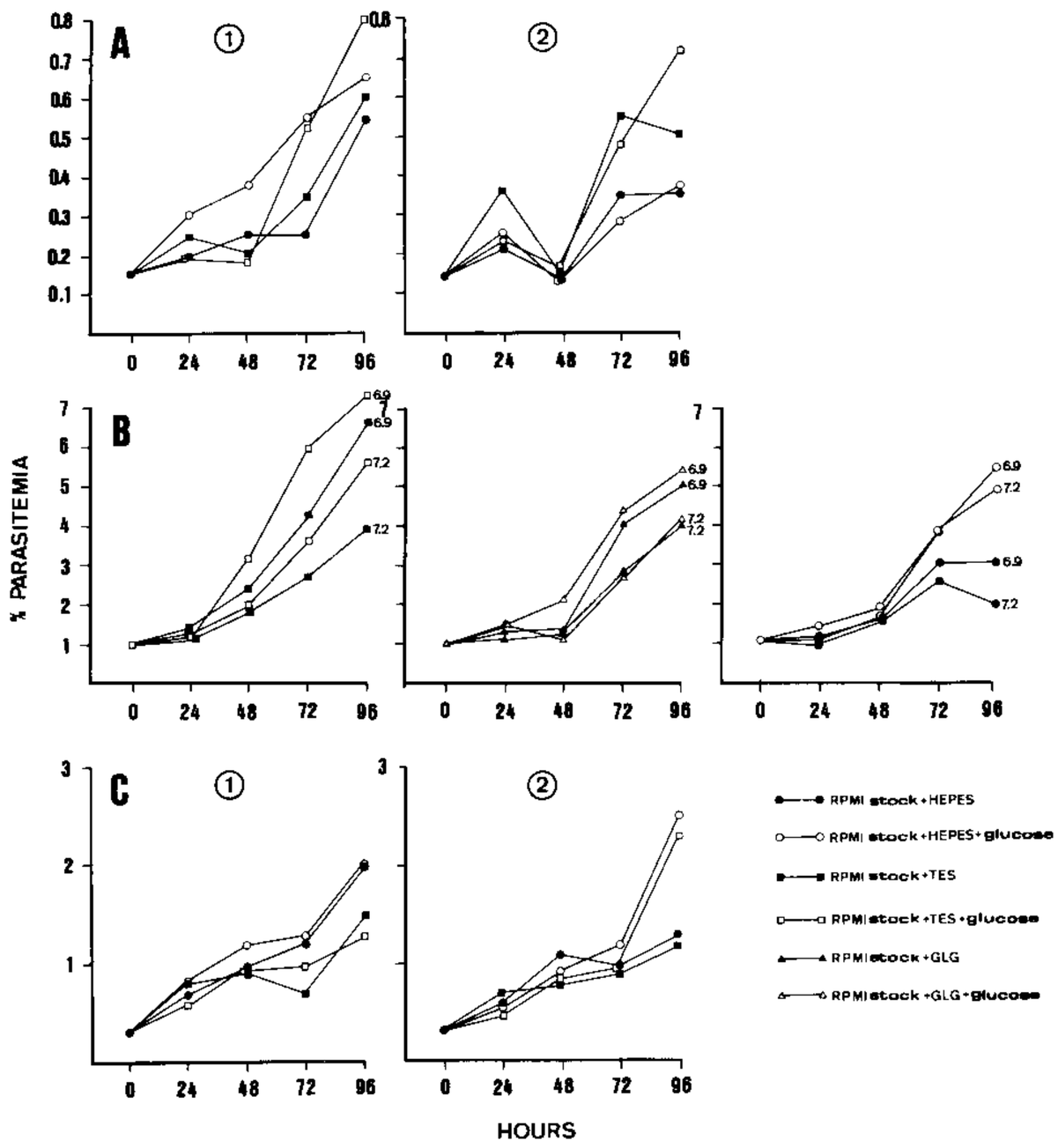

Fig. 1: some parameters affecting the growth of *Plasmodium falciparum* (FUP strain) within *Saïmiri* RBC in short term culture assay. A – **Medium supplemented with 10% *Saïmiri* serum:** the RPMI stock solution was buffered either with HEPES (30mM) or TES (20mM) to a final pH of 7.0. The medium was in some cases supplemented with 0.2% W/V glucose. Cultures were carried out in two days old RBC from (1) intact *Saïmiri* (2) splenectomized *Saïmiri*. B – **Medium supplemented with 20% AB^+ human serum:** the RPMI stock solution was buffered either with HEPES (3mM or 50mM), TES (2mM or 30mM) or glycylglycin (20mM or 150 mM) to a final pH of 6.9 or 7.2. The medium was in some cases supplemented with 0.2% W/V glucose. Cultures were carried out in fresh RBC from splenectomized monkeys. C – **Medium supplemented with A^+ human serum:** the RPMI stock solution was buffered either with HEPES (30mM) or TES (20mM) to a final pH of 7.0. The medium was in some cases supplemented with 0.2% W/V glucose. The RPMI stock solution was supplemented with (1) 20% human A^+ serum or (2) 10% human A^+ serum and 0.15% W/V proteose peptone. The final medium was buffered either with HEPES (30mM) or TES (20mM) to a final pH of 7.0. All cultures were performed within freshly collected RBC from a splenectomized *Saïmiri*. Each point is the mean value calculated from duplicate experiments.

During these passages, only exceptionally we observed degenerating parasites within erythrocytes, although numerous aborted schizonts and other late stages were observed at the same time in the medium.

The experiment 1 showed higher multiplication rates when cultures were initiated with RBC collected from an intact animal (Fig. 1 A). Taking into account the mechanical filtering function of the spleen, we wonder whether these results could not be related to an *in vivo* selection of a RBC sub-population more suitable for *in vitro* maintenance, as it is generally admitted that erythrocytes are filtered in the spleen on the basis of their decreasing deformability. We thus investigated in a third series of experiments, the influence of the spleen on *Saïmiri* RBC ability to support *in vitro P. falciparum* growth. Cultures of both Indo I and FUP *P. falciparum* strains were initiated simultaneously in RBC collected from a single animal, before and after its splenectomy and in human red blood cells as culture control.

TABLE I

Continuous culture assay of *Plasmodium falciparum* (FUP strain) within fresh or 15 day old *Saïmiri* RBC (in 24 well plates)

Days *in vitro*	Total dilution	Percentage of parasitemia°	
		Fresh RBC	15 days old RBC
0	1	0.3 ± 0.05	–
1		0.6 ± 0.1	–
2		0.9 ± 0.2	–
3		1.2 ± 0.1	–
4		2.5 ± 0.5	–
5	12.5	0.4 ± 0.1	0.3 ± 0.1
6		0.5 ± 0.1	0.7 ± 0.1
7		0.5 ± 0.1	0.7 ± 0.1
8		0.7 ± 0.1	0.8 ± 0.1
9		1.0 ± 0.1	0.9 ± 0.2
10	60	0.3 ± 0.05	0.4 ± 0.1
11		0.35 ± 0.05	0.45 ± 0.05
12		0.5 ± 0.1	0.7 ± 0.1
13		0.6 ± 0.1	0.6 ± 0.1
14	360	0.15 ± 0.05	0.20 ± 0.05
15		0.20 ± 0.05	0.30 ± 0.03
16		0.4 ± 0.1	0.55 ± 0.05
17	1800	0.11 ± 0.03	0.15 ± 0.02
18		0.06 ± 0.02	0.06 ± 0.02
19		< 0.01	< 0.01

° Each point is the mean value ± SD calculated from duplicates.

TABLE II

Effects of the splenectomy on the *Saïmiri* RBC ability to support the *Plasmodium falciparum* (FUP and Indo I strains) growth (in petri-dishes).

		Indo I strain					FUP strain				
		parasite count°				merozoite count°° in mature schizont	parasite count°				merozoite count°° in mature schizont
		parasites %	R %	T %	S %		parasites %	R %	T %	S %	
Human O^+ RBC	initial	0.7 ± 0.1	59 ± 2	27 ± 1	14 ± 1	14 ± 2	0.9 ± 0.1	42 ± 1	54 ± 2	4 ± 1	15 ± 1
	24 H	1.3 ± 0.2	86 ± 4	3 ± 1	11 ± 4	13 ± 2	1.3 ± 0.2	86 ± 3	8 ± 1	6 ± 1	14 ± 1
	48 H	1.6 ± 0.2	70 ± 7	21 ± 14	9 ± 7	12 ± 2	1.6 ± 0.2	75 ± 7	16 ± 1	9 ± 3	14 ± 2
	72 H	3.0 ± 0.4	66 ± 7	17 ± 7	17 ± 1	11 ± 1	3.4 ± 0.2	60 ± 7	17 ± 6	23 ± 1	11 ± 2
	96 H	3.8 ± 0.3	49 ± 4	43 ± 8	8 ± 5	11 ± 2	4.5 ± 0.4	38 ± 5	43 ± 7	19 ± 3	11 ± 2
RBC from S^+ *Saimiri*	initial	0.52 ± 0.02	57 ± 1	33 ± 4	10 ± 1	14 ± 2	0.7 ± 0.1	50 ± 2	50 ± 3	–	15 ± 1
	24 H	1.4 ± 0.3	69 ± 8	10 ± 4	21 ± 7	12 ± 7	1.4 ± 0.2	87 ± 3	5 ± 4	8 ± 4	14 ± 2
	48 H	1.5 ± 0.1	70 ± 4	21 ± 8	9 ± 7	9 ± 1	1.5 ± 0.1	83 ± 9	15 ± 2	2	14 ± 1
	72 H	2.7 ± 0.2	69 ± 7	16 ± 4	15 ± 3	10 ± 2	3.0 ± 0.3	70 ± 8	15 ± 3	15 ± 2	11 ± 2
	96 H	3.5 ± 0.3	68 ± 3	24 ± 2	8 ± 2	9 ± 2	4.2 ± 0.4	65 ± 2	30 ± 4	15 ± 1	10 ± 2
RBC from S^+ *Saimiri*	initial	0.57 ± 0.02	48 ± 3	30 ± 3	22 ± 1	14 ± 2	0.6 ± 0.1	53 ± 3	40 ± 2	7 ± 2	15 ± 1
	24 H	1.3 ± 0.2	68 ± 4	10 ± 3	22 ± 2	11 ± 1	1.1 ± 0.2	75 ± 7	10 ± 2	15 ± 5	15 ± 4
	48 H	2.1 ± 0.2	64 ± 7	18 ± 5	18 ± 5	11 ± 3	1.3 ± 0.1	71 ± 8	20 ± 3	9 ± 4	14 ± 3
	72 H	2.5 ± 0.4	68 ± 8	17 ± 2	15 ± 7	11 ± 2	2.7 ± 0.4	67 ± 6	17 ± 3	16 ± 5	12 ± 2
	96 H	4.1 ± 0.4	60 ± 3	29 ± 2	11 ± 1	9 ± 2	4.1 ± 0.3	58 ± 7	24 ± 3	18 ± 3	11 ± 2

° Each point is the mean value ± SD calculated from triplicates. R: ring, T: trophozoite, S: schizonte.
°° Each point is the mean value ± SD calculated from counts performed on 20 to 50 mature schizonts
S^+: intact *Saimiri*, S^-: splenectomized *Saïmiri*.

As shown in Table II, the origin of the RBC (from intact or splenectomized *Saïmiri*) did not influence the *in vitro* multiplication of the parasite. Percentages of parasitemia were quite similar for all culture systems (Table II), and merozoite number in mature schizonts decreased equally in time within human as well as *Saïmiri* RBC.

Thus the fluctuations of the multiplication rate observed in experiment 1 (Fig. 1, curves A) may relate to individual differences between *Saïmiri* donors, for example hemoglobin, or blood group rather than a RBC sub-population selection by the spleen of the intact animal.

DISCUSSION

The optimal conditions for *P. falciparum* culture in *Saïmiri* RBC, as defined from the present study; are the following:

The RPMI stock solution containing 0.2% W/V glucose, is supplemented with 20% heat inactivated FBS or human serum. Addition of 0.15% W/V proteose peptone with only 10% human serum represents an alternative possibility. The human sera should be previously selected in terms of their low hemagglutinin titer, and eventually fully absorbed on pooled *Saïmiri* RBC. Either TES or HEPES are added to the medium in order to obtain a final pH of 6.9-7.0.

With such general culture conditions, freshly collected or stored RBC, both from intact or splenectomized *Saïmiri*, were equally able to support the *in vitro* growth of two strains of *P. falciparum*. We have already demonstrated (Gysin & Fandeur, 1983) and here again give evidence that the FUP and Indo I strains adapted to *Saimiri sciureus* keep their infecting potential for human RBC. It is thus an unmodified parasite that we succeeded in cultivating *in vitro* in *Saïmiri* RBC. Two growth cycles were repeatedly obtained, but it was not possible to achieve continuous culture of the parasite in this type of RBC for longer than three weeks.

It has been suggested that the low success rate obtained in the culture of *P. knowlesi* in *Rhesus* monkey RBC was due to a reduction in the number of merozoites in the mature schizonts (Trigg & Shakespeare, 1976). This phenomenon definitely exists in the case of *P. falciparum* cultures in *Saïmiri* RBC, but this alone is not sufficient to explain our failure to establish a continuous culture in the RBC of this primate.

During our third series of experiments (Table II) we in fact observed an identical reduction of merozoite number in schizonts growing in human RBC wich otherwise allow an easy continuous culture of *P. falciparum*. More attention must be focused on the high number of extra-cellular late stages routinely observed in the culture medium, possibly in relation with an increase of osmotic fragility of the *Saïmiri* RBC when maintained under *in vitro* conditions.

The successful cultivation *in vitro* of the intracellular erythrocytic stages of the malaria parasites requires an environment which not only provides the correct growth factors for the parasite, but also ensures the right conditions for maintenance of the host-cell. Unfortunately, certain parameters selected in order to promote an optimal growth of the parasite, for example incubation at 37°C or a final pH of 7.0 of the culture medium, were found to increase fragility of the RBC (Murphy, 1962).

Another limiting factor for *P. falciparum* continuous culture in *Saïmiri* RBC is represented by an intrinsic character of these RBC, *i.e.* their reduced size (5-6 μm diameter) which might expose them to internal pressures higher than those sustained by human RBC (whose diameter is 6.7-7.7 μm). We can easily imagine that monkey RBC can support *in vivo* multiplication of the parasite in spite of their reduced size, while the same erythrocytes maintained *in vitro* in a synthetic medium not perfectly adapted to their osmotic requirements, cannot achieve complete maturation of all schizont stages. Thus an *in vitro* premature rupture of the IRBC may occur resulting in the low parasitemia found in our continuous culture experiments.

Nevertheless the regular obtention of two *in vitro* cycles of growth of *P. falciparum* in *Saïmiri* RBC, by permitting the realization of *in vitro* tests in parallel with *in vivo* experiments (Fandeur et al., 1984), opens new fields for the full utilization of the *Saïmiri sciureus*/*P. falciparum* as an experimental model for malaria research.

RESUMO

O crescimento e a multiplicação dos estágios eritrocíticos do *Plasmodium falciparum in vitro* foi estudado em cultivos com hemácias do *Saimiri sciureus* (macaco de cheiro). Foram investigados vários parâmetros tais como, origem das hemácias e suplementação de soro, tipo de tampão, influênia do pH final do meio, papel da proteose-peptona e da glicose adicionados.

A seleção das condições ideais de cultivo permitiram, de maneira reprodutível, a obtenção de crescimento do parasita durante dois ciclos nas hemácias do *Saimiri.* Nosso fracasso em estabelecer uma linhagem contínua de cultivo por mais de 19 dias poderia ser explicado pelo aumento dramático da fragilidade osmótica das hemácias do *Saimiri* relacionado com seu pequeno tamanho.

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