Plasmodium falciparum glutathione metabolism and growth are independent of glutathione system of host erythrocyte

Kodjo Ayi[a], Marina Cappadoro[a], Mario Branca[b], Franco Turrini[a], Paolo Arese[a,*]

[a] *Dipartimento di Genetica, Biologia e Biochimica, Università di Torino, Via Santena 5 bis, 10126 Turin, Italy*
[b] *Dipartimento di Chimica, Università di Sassari, Sassari, Italy*

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Abstract ***Plasmodium falciparum*** **parasites grew normally in glutathione (GSH)-depleted normal and G6PD-deficient (Mediterranean variant) erythrocytes (RBC). Growth inhibition was observed only at less than approximately 6–12% residual GSH. Parasites studied separately with the Sendai virus technique synthesized GSH de novo and regenerated reduced GSH 10–20 times faster than non-parasitized RBC. Electron spin resonance measurement of Tempol reduction indicated that the ability to reduce free radicals was restricted to the parasite. The marked efflux of oxidized GSH was mainly derived from the parasite. In conclusion, parasites are endowed with powerful and host-independent mechanisms which de novo synthesize or regenerate GSH and allow undisturbed parasite development in GSH-depleted RBC.**

Key words: Glutathione; Malaria (*Plasmodium falciparum*); G6PD deficiency (Mediterranean variant); Anti-oxidant defense

1. Introduction

Reduced glutathione (GSH) plays important roles in *Plasmodium falciparum*-parasitized erythrocytes (RBC): it protects host RBC against oxidant stress [1,2], modulates parasite-induced RBC membrane modifications and heme catabolism [3,4], and seems to affect parasite growth [5,6]. Parasite growth has been reported in some studies to be inhibited in RBC mutants with low GSH levels, such as in glucose-6-phosphate dehydrogenase (G6PD)-deficient RBC [5–8], while in other studies parasite growth appeared to be normal in G6PD-deficient RBC [5,9]. Growth inhibition data [5,9], and results by Roth et al. [10] showing that GSH was extremely low in parasitized G6PD-deficient RBC, seemed to imply that parasite GSH was dependent on the host's glutathione system.

In view of the importance of GSH for both parasite and host cell, and in front of the above controversies, we analyze here the separate contributions of host and parasite to GSH homeostasis. At variance with previous reports [5,9,10], we show that parasite growth is vastly independent of GSH levels in the host cell. We also show that the parasite is endowed with a powerful GSH-synthesizing and GSH-regenerating system both independent of host cell metabolism.

2. Materials and methods

2.1. Materials

Culture media, diazenedicarboxylic acid bis(*N*,*N*-dimethylamide) (diamide), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), NADPH, GSH and glutathione reductase were from Sigma; [1-^{14}C]glycine (0.2 mCi/ml) was from Amersham; Percoll was from Pharmacia; monobromobimane was from Calbiochem; Diff-Quik parasite stain was from Baxter Dade AG, Dudingen, Switzerland; 2,2,6,6-tetramethylpiperidin-1-oxyl-4-ol (Tempol) was prepared according to [11]; Sendai virus was a kind gift of Prof. H. Ginsburg, Jerusalem. All other reagents were purchased from common commercial sources.

2.2. Parasite culture and cell treatments

G6PD-deficient and normal RBC were isolated from freshly drawn blood of healthy, non-hemolytic males of Sardinian origin (Sassari province), hemizygous for G6PD deficiency (Mediterranean variant with 1–3% residual G6PD activity), and of normal age-matched male controls, respectively. Deficient and normal RBC were parasitized with *P. falciparum* strain FCR-3 and kept in continuous culture according to Trager and Jensen [12]. Malaria cultures were free of mycoplasma contamination as checked by PCR assay as indicated [13]. Trophozoite-stage-parasitized RBC (P-RBC) were isolated from non-synchronized cultures by Percoll gradient [14]. To assess total parasitemia and the relative contributions of ring-stage- and trophozoite-stage-parasitized RBC, slides were prepared from cultures 14–18 h (ring stage) and 34–38 h (trophozoite stage) after inoculation, stained with Diff-Quik parasite stain and 400–1000 cells examined microscopically. Significance of differences in parasitemia between normal and deficient parasitized RBC was assessed by *t*-test for paired samples.

2.3. Depletion and regeneration of GSH

GSH was depleted before inoculation by oxidation with diamide [15] or by alkylation with CDNB treatment [16]. Diamide (0.1–0.5 mM) and CDNB (0.5–2.0 mM) were added to RBC suspensions (1% hematocrit) for 5 min at 4°C or 15 min at room temperature, respectively. After treatment, RBC were washed 4 or 6 times with a 30-fold excess of phosphate-buffered saline with 10 mM glucose at pH 7.4 (PBS-G), respectively, and parasite inoculation performed as indicated [17]. GSH regeneration studies were performed with washed RBC, P-RBC and isolated parasites (see Section 2.4) incubated with or without 0.1 mM diamide at 10^9 cells/ml in PBS-G for 5 min at 4°C. Thereafter cells were sedimented with a refrigerated microcentrifuge and resuspended to the original cell number with pre-warmed (37°C) PBS-G. Samples were taken time-dependently and GSH measured as described [18].

2.4. Measurement of overall GSH levels and compartment analysis of GSH

Overall GSH levels were measured in washed non-parasitized RBC and P-RBC as indicated [18]. GSH was also measured separately in the host and parasite compartments after permeabilization of the RBC membrane by Sendai virus treatment. Sendai virus specifically lyses RBC membrane causing efflux of intracellular molecules with a molecular mass lower than approximately 50 000 Da, but leaves the parasite membrane intact [19]. P-RBC (trophozoite stage) were incubated at 10^9 cells/ml in PBS-G supplemented or not with 10 μl Sendai virus solution, containing 40–100 μg virus protein/ml, equivalent to 800–1200 hemagglutination units for 20 min at 37°C. Thereafter cells were sedimented with a refrigerated microcentrifuge and GSH was assayed in the supernatant (host cytosol compartment) and in the pellet (parasite compartment) as described [18]. The pellet of Sendai virus-treated non-parasitized RBC was totally devoid of GSH (not shown). Measurement of overall GSH levels and GSH compartment analysis was also performed in GSH-depleted P-RBC. Before infec-

*Corresponding author. Fax: (39) (11) 6635663.
E-mail: arese@molinette.unito.it

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tion with the parasite, RBC were treated or not with 1.5 mM CDNB. The treatment irreversibly alkylated approximately 85–88% of total glutathione without impairing parasite growth (see Section 2.3). After infection, P-RBC (trophozoite stage) were treated or not with Sendai virus and compartment analysis performed as described above.

2.5. Measurement of oxidized glutathione (GSSG) efflux

GSSG efflux from RBC through a specific transport system is gradient-dependent [20] and, therefore, maximized by GSH oxidation produced by diamide treatment. Washed RBC, P-RBC and isolated parasites were incubated at 10^9 cells/ml in PBS-G supplemented or not with 0.1–0.5 mM diamide for 20 min at 37°C. GSH-depleted cells were sedimented at selected time points with a refrigerated microcentrifuge and GSSG efflux rate measured by assaying GSSG in the collected supernatants as described [21].

2.6. Measurement of de novo GSH synthesis

Washed RBC, P-RBC and isolated parasites were incubated at 10^8 cells/ml in PBS-G supplemented or not with [1-^{14}C]glycine (2 μCi/ml) for 3 h at 37°C. Thereafter cells were supplemented or not with 1 mM diamide for 5 min at 4°C and washed twice with PBS-G. Resuspended cells were then incubated in PBS-G supplemented or not with [1-^{14}C]glycine (2 μCi/ml) for 2 h at 37°C. Thereafter GSH was extracted as described [18] and labeled with monobromobimane (8 mM final) for 2 h at room temperature in the dark. The resulting fluorescent GSH-bimane adduct was separated by HPLC [22] and quantified using authentic GSH-bimane adduct as a standard. The incorporation of [1-^{14}C]glycine in GSH was determined by measuring the radioactivity of the peak containing the GSH-bimane adduct.

2.7. Electron spin resonance (ESR) measurement of Tempol reduction

Washed RBC, P-RBC and isolated parasites were incubated at 10^9 cells/ml in PBS-G supplemented or not with 0.1 mM Tempol. First-rate constants of Tempol reduction were calculated from the decay of ESR signal as described [11]. ESR spectra were recorded on a Bruker 220D equipped with a variable temperature accessory.

3. Results

3.1. Growth of malaria parasites in GSH-depleted RBC

Dependence of the growth of malaria parasites on GSH levels was studied with GSH-depleted P-RBC. GSH was irreversibly depleted by two different treatments performed before RBC infection. GSH was specifically alkylated by CDNB [16] in normal RBC, or oxidized by diamide [15] in G6PD-deficient RBC, which were unable to reduce oxidized glutathione during the incubation time (not shown). Table 1 shows that depletion of GSH by alkylation with CDNB to approximately 12% residual GSH did not significantly inhibit parasite growth in normal RBC. Only complete alkylation of GSH inhibited parasite growth (not shown), probably because CDNB doses higher than 2 mM caused consistent RBC damage [23] and lysis, and could therefore be toxic to the parasite as well. In G6PD-deficient P-RBC, extensive irreversible oxidation of GSH by diamide down to approximately 6% residual GSH did not significantly affect parasite growth.

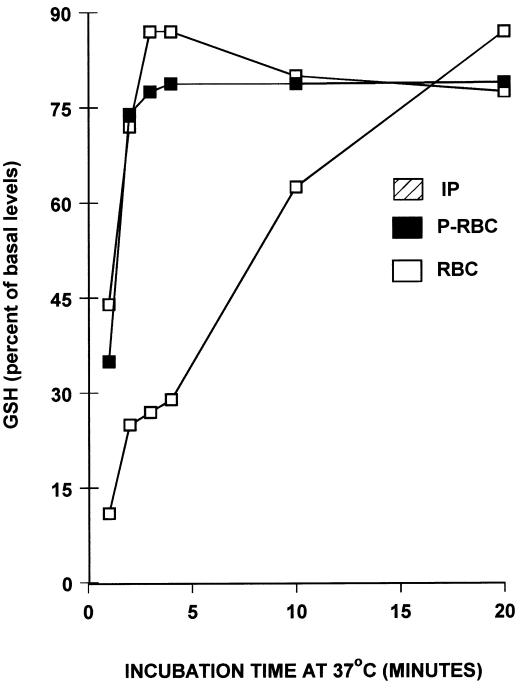

Fig. 1. GSH regeneration in non-parasitized RBC (RBC), parasitized RBC (P-RBC) and isolated parasites (IP) after GSH oxidation by diamide (0.1 mM final) treatment. Values are expressed as percent of the respective basal GSH levels measured before diamide treatment. Mean values of triplicate experiments.

3.2. Overall GSH levels and compartment analysis of GSH

As shown in Table 2, the overall level of GSH was diminished by approximately 63% in P-RBC compared to non-parasitized controls. GSH was measured separately in the host and parasite compartment after permeabilization of the RBC membrane by Sendai virus treatment. The GSH level was only apparently higher in the parasite compartment (633 ± 138 vs. 166 ± 57 nmol/10^{10} cells). In fact, taking into account the relative volume occupied by trophozoites (approximately 80% of total cellular volume in P-RBC), the distribution of GSH appears to be homogeneous in the host and parasite compart-

Table 1
Parasite growth in GSH-depleted normal and G6PD-deficient RBC

	GSH at inoculum (% of control)	Parasitemia	
		Ring stage 24 h)	Trophozoite stage (48 h)
Normal RBC			
0.5 mM CDNB	71 ± 15	115 ± 34	104 ± 24
1 mM CDNB	28 ± 8	124 ± 29	103 ± 20
1.5 mM CDNB	12 ± 5	105 ± 22	80 ± 28
G6PD-deficient RBC			
0.5 mM diamide	6 ± 4	78 ± 37	90 ± 5

GSH was depleted by treating normal and G6PD-deficient RBC with CDNB and diamide, respectively. GSH-depleted and control RBC were subsequently utilized for malaria culture. GSH values are percent values of control levels measured in normal or G6PD-deficient RBC before depleting treatments. Control GSH levels were 1950 ± 320 nmol/10^{10} cells (normal RBC) and 1430 ± 180 (G6PD-deficient RBC) (mean ± S.D. of triplicate experiments). Parasitemias are percent values of control parasitemias measured in normal or G6PD-deficient RBC at the ring or trophozoite stage. Control parasitemias were 3.8 ± 0.7% parasitized RBC (ring stage) and 3.1 ± 0.5% parasitized RBC (trophozoite stage) after inoculation (mean ± S.D. of triplicate experiments). Differences in parasitemias measured in control and GSH-depleted RBC were never significant.

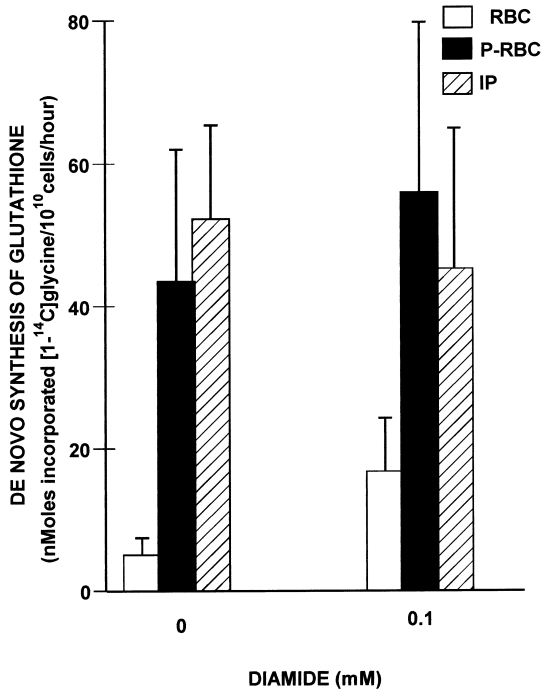

Fig. 2. De novo GSH synthesis in non-parasitized RBC (RBC), parasitized RBC (P-RBC) and isolated parasites (IP) in basal conditions and after GSH oxidation by diamide treatment. GSH synthesis is expressed as nmol of incorporated [^{14}C]glycine/10^{10} cells/h. Mean values ± S.D. of triplicate experiments. Bars indicate standard deviation.

ments. GSH analysis was also performed in GSH-depleted RBC. Treatment of RBC with CDNB (1.5 mM final) before parasite inoculation irreversibly alkylated approximately 85% of total GSH (Table 2). CDNB-treated RBC supported normal parasite development (see Section 3.1) and showed approximately 40% increase in overall GSH level at trophozoite stage. Since no measurable regeneration of GSH was observed in CDNB-treated non-parasitized RBC after 34–38 h incubation, parasites were evidently able to synthesize GSH. Compartment analysis indicated a homogeneous distribution of GSH also in CDNB-treated P-RBC.

3.3. GSH regeneration and de novo synthesis in malaria parasite

As shown in Fig. 1, isolated parasites were able to regenerate GSH at a very fast rate: less than 2 min was necessary to essentially restore basal GSH levels. P-RBC showed a rate of GSH regeneration very similar to that observed in isolated parasites. RBC regenerated GSH at a much lower rate and approximately 20 min was necessary to restore the basal GSH levels. These data indicate that isolated parasites possess efficient mechanisms to regenerate GSH and that the GSH-regenerating activity in P-RBC is mainly due to the parasite contribution.

To test the ability of P-RBC to utilize their GSH to metabolize oxidant species we monitored their capability to reduce Tempol, a membrane-permeable mild oxidant which becomes non-reactive upon enzymatic coupling to intracellular GSH [11]. Using ESR measurement, first-rate constants (KT) of Tempol regeneration were observed in all conditions considered. KT was 0.061 ± 0.012 in control RBC, 0.022 ± 0.0025 in P-RBC and 0.019 ± 0.0037 in isolated parasites (mean values ± S.D., $n = 3$). KT was almost identical in P-RBC and isolated parasites, confirming that the ability to reduce oxidant species appears to be limited to the parasite. Moreover, the first-order kinetics of Tempol decay indicates that parasites were able to keep constant GSH levels during the experimental period even under moderate oxidant stress.

In addition to fast GSH regeneration, the parasites were endowed with powerful de novo GSH synthesizing ability. Fig. 2 shows that P-RBC and isolated parasites, but not RBC, displayed a remarkable GSH synthetic activity even in the absence of oxidative treatment by diamide. Diamide treatment strongly stimulated de novo synthesis in RBC but had no effect in P-RBC and isolated parasites.

3.4. Efflux of GSSG from P-RBC and isolated parasites

GSSG permeates across the RBC membrane through a specific channel and GSSG efflux is maximized upon extensive oxidation of GSH [20]. The relatively low level of GSH in P-RBC and in isolated parasites in the presence of intense de novo GSH synthesis may suggest GSSG efflux from P-RBC. Indeed, the efflux rate of GSSG from untreated P-RBC and isolated parasites was much higher than from control RBC (see Fig. 3). To test whether RBC membrane was a rate-limiting step for GSSG efflux from P-RBC, GSH was extensively oxidized by diamide treatment. While GSSG efflux rate from P-RBC was not influenced by diamide treatment, diamide-treated isolated parasites showed a 2–4-fold GSSG efflux rate compared to diamide-treated P-RBC (Fig. 3).

4. Discussion

In this work we show normal growth of *P. falciparum* in GSH-depleted normal and G6PD-deficient (Mediterranean variant) RBC. Impairment of parasite growth was observed only below approximately 6–12% residual GSH. The ability of malaria parasites to grow in GSH-depleted RBC was evidently connected with the existence of an autonomous and efficient glutathione system independent of host metabolism.

Table 2
Overall GSH levels in RBC and P-RBC, and compartment analysis of GSH in P-RBC

Treatment	Overall levels		Compartment analysis	
	RBC	P-RBC	Parasite	RBC
None	1822 ± 220	681 ± 152	166 ± 57	633 ± 138
CDNB (1.5 mM)	282 ± 87	459 ± 119	110 ± 41	307 ± 76

Overall levels of GSH were measured in intact RBC and P-RBC treated or not with CDNB (1.5 mM final) before parasite infection. Compartment analysis of GSH was performed in isolated parasites (parasite compartment) and host RBC cytosol (RBC compartment) from Sendai virus-treated P-RBC, treated or not with CDNB (1.5 mM final) before parasite infection. GSH levels are expressed as nmol GSH/10^{10}cells. Mean values ± S.D. of triplicate experiments.

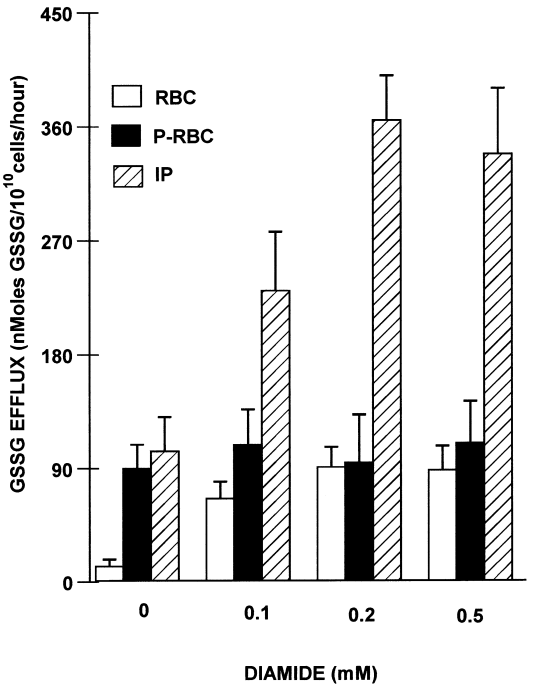

Fig. 3. Rate of GSSG efflux in non-parasitized RBC (RBC), parasitized RBC (P-RBC) and isolated parasites (IP) in basal conditions (no treatment) and after GSH oxidation by diamide treatment. GSSG efflux is expressed as nmol GSSG/10^{10} cells/h. Mean values ± S.D. of triplicate experiments. Bars indicate standard deviation.

The parasite was indeed capable of de novo GSH synthesis at a high rate, and was able to extensively regenerate GSH in less than 2 min, compared to 20 min required by non-parasitized RBC.

Here we also show that the parasite membrane was highly permeable to GSSG, compared to the membrane of parasitized or non-parasitized RBC. Thus, the host cytoplasm behaves as a sink for parasite GSSG, potentially available for back-reduction to GSH by NADPH-dependent glutathione reductase of the host. Parasite-produced GSSG is expected to pose a critical oxidative stress to G6PD-deficient RBC, by competing with endogenous GSSG for NADPH, insufficiently provided by the low residual G6PD activity.

The glutathione system in malaria and the relationship between GSH and parasite growth have been the object of several studies (for review, see [2]). Most studies indicated impaired parasite growth in GSH-oxidized or G6PD-deficient RBC characterized by low levels of GSH and an impaired capacity to reduce GSSG [5–8]. Our data are at variance with those studies and agree with the no-difference data by Pasvol and Wilson [9] and Fritsch et al. [24]. Studies showing impaired growth in G6PD-deficient RBC [5–8] were performed with the candle-jar method [12], where a burning candle was used to lower oxygen tension in the airtight incubator. Toxic fumes from the extinguishing candle may preferentially upset the more delicate deficient RBC and possibly explain the discrepancy. Low levels of GSH have been described in *P. falciparum* P-RBC by others [25], where high GSH levels had been described previously in parasitized RBC in murine malaria [26] and increased stability of GSH remarked by Roth et al. [27] in *P. falciparum*-parasitized RBC. Various interpretations were offered for the 'unexpected' [27] result, but no clear evidence was provided for a de novo synthetic ability of the isolated parasite, shown here for the first time.

The present results are of interest in two respects. First, they indicate that the parasite is independently endowed with highly efficient anti-oxidant mechanisms capable of de novo synthesis of GSH, and GSH regeneration through parasite-encoded G6PD [28] and glutathione reductase [29]. As a consequence, the parasite is a well-protected target for oxidant drugs. Only oxidants targeted to specific parasite components are able to destroy the parasite at acceptably low concentrations. One good example is artemisinin, a stable endoperoxide that binds to and is activated by the clustered heme of malarial pigment hemozoin [30].

Secondly, the present results suggest that non-parasite-targeted oxidants may not be effective as direct parasite killers. Instead, the combined oxidant stress provided by the parasite and by the exogenous oxidant, unable to impair the development of parasite, may nevertheless target the parasitized RBC for destruction by the macrophage system of the host. This indirect parasite killing mechanism is particularly effective in parasitized G6PD-deficient RBC, where enhanced phagocytic removal of ring-parasitized, G6PD-deficient RBC has been observed (Cappadoro et al., unpublished results). Selective removal of parasitized G6PD-deficient RBC may explain older data by Luzzatto et al. [31] showing a much higher frequency of circulating parasitized G6PD-normal RBC compared to parasitized G6PD-deficient RBC in heterozygotes that carry a double population of normal and deficient RBC in their blood.

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References

[1] Hunt, N.H. and Stocker, R. (1990) Blood Cells 16, 499–526.
[2] Ginsburg, H. and Atamna, H. (1994) Parasite 1, 5–13.
[3] Atamna, H. and Ginsburg, H. (1995) J. Biol. Chem. 270, 24876–24883.
[4] Turrini, F., Ginsburg, H., Bussolino, F., Pescarmona, G.P., Serra, M.V. and Arese, P. (1992) Blood 80, 801–808.
[5] Miller, J., Golenser, J., Spira, D.T. and Kosower, N.S. (1984) Exp. Parasitol. 57, 239–247.
[6] Golenser, J., Miller, J., Spira, D.T., Kosower, N.S., Vande Waa, J.A. and Jensen, J.B. (1988) Trop. Med. Parasitol. 39, 272–276.
[7] Usanga, E.A. and Luzzatto, L. (1985) Nature 313, 793–795.
[8] Roth Jr., E.F., Raventos-Suarez, C., Rinaldi, A. and Nagel, R.L. (1983) Proc. Natl. Acad. Sci. USA 60, 298–299.
[9] Pasvol, G. and Wilson, R.J.M. (1982) Br. Med. Bull. 38, 133–140.
[10] Roth Jr., E.F., Schulman, S., Vanderberg, J. and Olson, J. (1986) Blood 67, 827–830.
[11] Branca, M., Denurra, T. and Turrini, F. (1988) Free Radical Biol. Med. 5, 7–11.
[12] Trager, W. and Jensen, J.B. (1976) Science 193, 673–675.
[13] Turrini, F., Giribaldi, G., Valente, E. and Arese, P. (1997) Parasitol. Today 13, 367–368.
[14] Rivadeneira, E.M., Wasserman, M. and Espinal, C.T. (1983) J. Protozool. 30, 367–370.
[15] Kosower, N.S., Kosower, E.M., Wertheim, B. and Correa, W.S. (1969) Biochem. Biophys. Res. Commun. 37, 593–596.
[16] Awasthi, Y.C., Grag, H.S., Dao, D.D., Partridge, C.A. and Srivastava, S.K. (1981) Blood 58, 733–738.

[17] Lambros, C.J. and Vanderberg, J. (1979) J. Parasitol. 65, 418–420.
[18] Beutler, E. (1975) A Manual of Biochemical Methods, pp. 12–113, Grune and Stratton, New York.
[19] Kanaani, J. and Ginsburg, H. (1989) J. Biol. Chem. 264, 3194–3199.
[20] Beutler, E. and Dale, G.L. (1989) in: Coenzymes and Cofactors. Glutathione. Chemical, Biochemical and Medical Aspects, part B (Dolphin, D., Avramovic, O. and Poulson, R., Eds.), pp. 291–317, Wiley, New York.
[21] Owens, C.W.I. and Betcher, R.V.I. (1965) Biochem. J. 94, 705–711.
[22] Newton L, G., Dorian, R. and Fahey, R.C. (1981) Anal. Biochem. 114, 383–387.
[23] Chiu, D.T.Y., Lai, K.-M., Xu, C.-M., Liour, S.S.S., Lee, J. and Liu, T.Z. (1993) Exp. Hematol. 21, 114–118.
[24] Fritsch, B., Dieckmann, A., Menz, B., Hempelmann, E., Fritsch, K.-G., Fritsch, G. and Jung, A. (1987) Parasitol. Res. 73, 515–517.
[25] Picard-Maureau, A., Hempelmann, E., Krämer, G., Jackisch, R. and Jung, A. (1975) Trop. Med. Parasitol. 26, 405–416.
[26] Eckman, J.R. and Eaton, J.W. (1979) Nature 278, 754–756.
[27] Roth Jr., E.F., Raventos-Suarez, C., Perkins, M. and Nagel, R.L. (1982) Biochem. Biophys. Res. Commun. 109, 355–362.
[28] O'Brien, E., Kurdi-Haidar, B., Wanachiwanawin, W., Carvajal, J.L., Vulliamy, T., Cappadoro, M., Mason, P.J. and Luzzatto, L. (1994) Mol. Biochem. Parasitol. 64, 313–326.
[29] Krauth-Siegel, R.L., Müller, J.G., Lottspeich, F. and Schirmer, R.H. (1996) Eur. J. Biochem. 235, 345–350.
[30] Hong, Y.-L., Yang, Y.-Z. and Meshnick, S.R. (1994) Mol. Biochem. Parasitol. 63, 121–128.
[31] Luzzatto, L., Usanga, E. and Reddy, S. (1969) Science 164, 839–841.