Compared Reactivities of Trypanothione and Glutathione in Conjugation Reactions

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In order to compare the non-enzymatic capacities of the xenobiotic conjugation of trypanothione (a spermidine–glutathione conjugate unique to kinetoplastidae) and glutathione, the reactivity of their respective thiols was investigated. The acido-basic properties of both compounds and their nucleophilicity toward Ellman's reagent and 1-chloro-2,4-dinitrobenzene were studied. Our results show that although glutathione is a better nucleophile than trypanothione, the latter is more reactive because it is more ionized in a large pH range. This pH range likely includes the pH to which such conjugation reactions are expected to happen *in vivo*. Thus, the better conjugation capacity of trypanothione could make it the cornerstone for the xenobiotic detoxication of trypanosomatidae.

Keywords trypanothione; glutathione; xenobiotic detoxication; thiol reactivities; *Trypanosoma cruzi*

Glutathione S-conjugates play an essential role in the physiological mechanisms involved in the survival of the cell. They are the products of the nucleophilic addition of glutathione on a wide variety of electrophiles catalyzed by glutathione S-transferases (GST) (EC 2.5.1.18), and represent the most important pathway for the detoxication of endogeneous and xenobiotic electrophilic substances. These glutathione S-conjugates are less reactive and more polar than the initial electrophilic molecules and can therefore be more easily eliminated.[1] Some glutathione S-conjugates serve as mediators, for example cysteine leucotrienes (LTC4),[2] whose chemical structure represents a convenient form for export *via* the ATP-dependent glutathione S-conjugate export pump.[3,4] These conjugates can also be formed in a reaction which is chemically reversible under physiological conditions and can serve *in vivo* as transporters for biochemically important electrophilic compounds.[5]

All organisms contain at least one low molecular weight thiol in high amounts available for this conjugate formation. The far more common compound is glutathione, but some organisms, such as kinetoplastidae, have analogs of glutathione, and instead use trypanothione (N^1,N^8-bis(glutathionyl)spermidine) (Fig. 1). This unusual dithiol ($T(SH)_2$) is essential for reducing glutathione disulfide (GSSG).[6] Indeed, trypanosomatidae lack glutathione reductase (EC 1.6.4.2) and possess instead trypanothione reductase (EC 1.6.4.8), an enzyme unique to these organisms, which regenerates $T(SH)_2$ from trypanothione disulfide ($T(S)_2$). Therefore $T(SH)_2$ can represent the main parasitic molecule in defense against reactive oxygen species.[7,8] Its potential role in xenobiotic detoxication has been suspected for a long time.

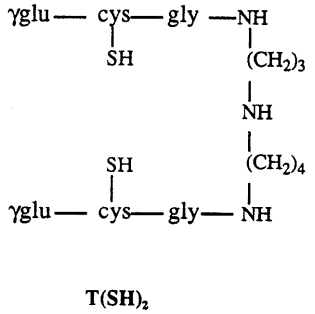
In 1981 the purification of a protein having a low GST activity was described in *Trypanosoma cruzi*.[9] However, we could not detect any significant GST or trypanothione S-transferase activity in fresh lysates of *T. cruzi*. The non-enzymatic capacities of the conjugation of trypanothione were thus studied and compared to those of glutathione. The experimental conditions were chosen from the physiological data known on *T. cruzi*. On the one hand, given that the reactivity of thiols for conjugation reactions is strongly related to their acido-basic properties, the ionization profiles of $T(SH)_2$ *versus* GSH were determined. On the other hand, since these conjugation reactions proceed through a *SN*2 mechanism, two electrophiles were used: Ellman's reagent[10] and 1-chloro-2,4-dinitrobenzene (CDNB). These reagents are used, respectively, to study thiol-disulfide exchange and to evaluate glutathione S-transferase activities.[11]

Experimental

Materials CDNB, Ellman's reagent (Ell-S-S-Ell: 5,5′-dithiobis(2-nitrobenzoic acid)) and GSH were obtained from Aldrich. $T(S)_2$ was synthetized according to Fauchet *et al.*[12] pH was determined using an Orion Research model 601 A pH meter, and UV spectra were measured using a Uvikon 930 spectrophotometer (Kontron instruments). Glutathione reductase (GR) from bovine intestinal mucosa was purchased from Sigma. Trypanothione reductase (TR) was purified as previously described.[13]

Spectrophotometric Titration of GSH and $T(SH)_2$ The study was carried out according to the protocol of Benesch.[14] The buffer used was a mixture of orthophosphoric and boric acid at a concentration of 0.02 M in each acid. The pHs were adjusted with NaOH. All the pH measurements and the corresponding spectrophotometric readings were made at 28 °C. For spectrophotometric measurements, 10 μl of a 20 mM solution of thiol was added to 1 ml of the buffer solution. The absorption spectrum was determined immediately after mixing, using the corresponding buffer as a blank.

Kinetics Reaction with Ellman's Reagent: Ellman's reaction was studied according to Wilson's protocol[15]: 0.04 M sodium acetate buffer, pH 4.7, 1 M KCl, 40 μM Ellman's reagent, and 0.25 to 1 mM thiol



$T(SH)_2$

Fig. 1. Structure of Reduced Trypanothione $T(SH)_2$

concentration. The reaction was studied at a pH value far below their pK_a so that the reaction could proceed at a measurable rate. The thiol concentrations were verified by titration with Ellman's reagent in 0.1 M phosphate buffer, pH 8. The reaction was studied spectrophotometrically at 412 nm and 28 °C for 1 min. The pH of the solution was measured after each run.

Addition on CDNB: The reaction was studied under the following conditions: 0.1 M phosphate buffer, pH 6.8 and 7.8, 250 μM CDNB, and 0.1 to 1 mM thiol concentration. The formation of the conjugate CDNB-thiol was followed at 340 nm and 28 °C for 2 min. The molecular extinction coefficients used for the conjugates were $\varepsilon = 9600\,cm^{-1}\,M^{-1}$ for GSH[11] and $\varepsilon = 9900\,cm^{-1}\,M^{-1}$ for $T(SH)_2$ per thiol group, respectively.

Results

Ionization Profiles of $T(SH)_2$ and GSH It has been established that thiols react predominantly as thiolates and that the nucleophilicity of a thiolate anion depends on its basicity.[1] As GSH and $T(SH)_2$ possess numerous ionizable functions, a direct study of their acido-basic properties is difficult. The method of Benesch[14] allowed us to quantify the percentage of thiolate according to the pH. For pHs between 5.5 and 9.5, $T(SH)_2$ (Fig. 2, curve A) is more ionized than GSH (Fig. 2, curve B). For pHs less than 5.5, neither $T(SH)_2$ nor GSH are sufficiently ionized to possess a significant reactivity. For pHs above 9.5, both species are almost completely ionized; therefore, the thiol reactivity in nucleophilic addition depends no more on thiolate quantity but on their intrinsic nucleophilicity.

At pH 7.4, close to the physiological value, the percentage of ionization of thiolate function is 1% for glutathione and 15% for trypanothione. Given the dithiol structure of $T(SH)_2$, the quantity of thiolate is therefore thirty-fold higher for $T(SH)_2$ than for GSH. This ratio decreases from pH 5.5 to 9.5.

Kinetics of Reduction of Ellman's Reagent The reduction of disulphide bonds is known to proceed through the thiolate ion.[1] The thiol-disulfide exchange with Ellman's reagent (Chart 1) enabled us to easily follow this kind of reaction using spectrophotometry. Previous studies[15,16] indicate that thiolate-disulfide exchange is a mechanistically simple S_N2 displacement reaction.

For monothiols, it is shown that $k_2 < k_1$. Thus, for reaction times of less than 1 min, any contribution to the formation of Ell-S$^-$ from (iii) in Chart 1 can be neglected. The rate expression of Ell-S$^-$ formation is:

$$v = \frac{d[\text{Ell-S}^-]}{dt} = k_1[\text{RS}^-][\text{Ell-S-S-Ell}] \quad (1)$$

The large excess of thiol enables us to write $[RS^-] = [RS^-]_0$

Equation 1 simplifies to:

$$v = \frac{d[\text{Ell-S}^-]}{dt} = k_{1\,obs}([\text{Ell-S-S-Ell}]_0 - [\text{Ell-S}^-]_t) \quad (2)$$

with $k_{1\,obs} = k_1[RS^-]_0 = k_1 \dfrac{K_a}{K_a + [H_3O^+]}([RS^-] + [RSH])_0$

For dithiols, the reaction (iii) is essentially intramolecular. In a dilute solution, the intermolecular thiol-disulphide exchange does not compete with the intramolecular reaction. A rate equation of the same form

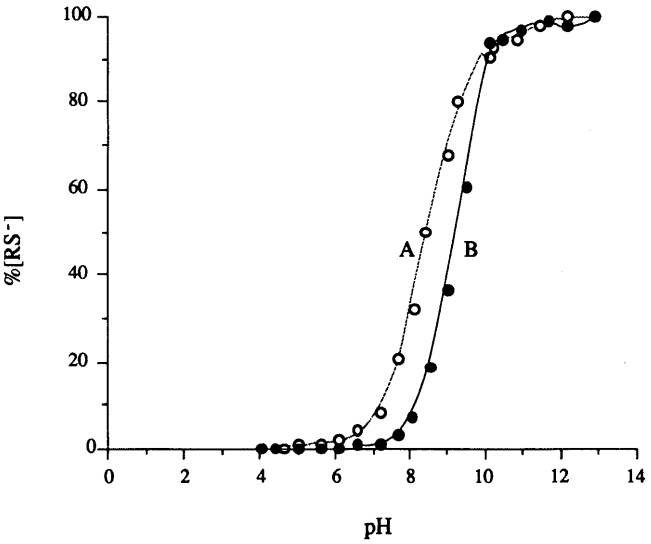


Fig. 2. Spectrophotometric Titration Curves of Thiol Functions
A, trypanothione 200 μM; B, glutathione 200 μM.

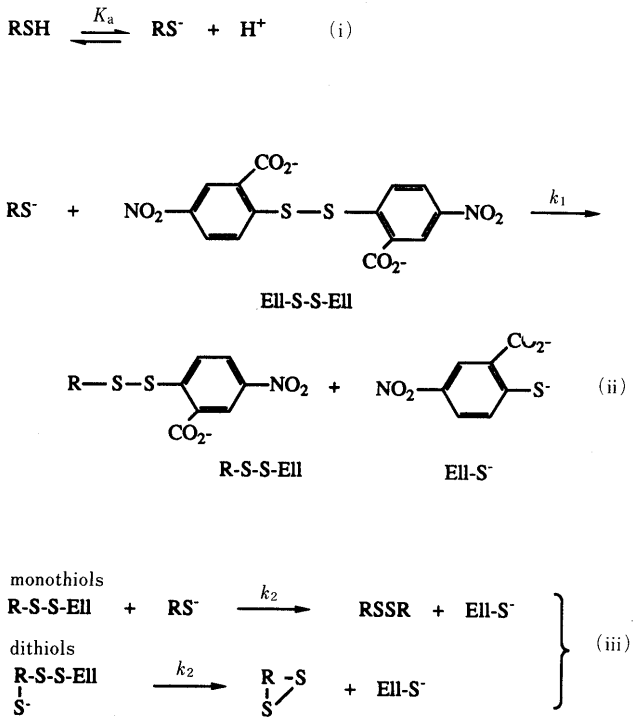


Chart 1. Thiol-Disulfide Exchange with Ellman's Reagent

as Eq. 1 can be written[16]:

$$v = \frac{d[\text{Ell-S}^-]}{dt} = 2k_1[S^-][\text{Ell-S-S-Ell}] \quad (3)$$

with $[S^-] = [^-SRS^-] + [^-SRSH]$

The factor of 2 in Eq. 3 reflects the assumption that the reaction (ii) is rate limiting and the production of a second equivalent of Ell-S$^-$ by reaction (iii) follows rapidly, once the intermediate disulfide HSR-S-S-Ell is formed. $^-SRS^-$ and ^-SRSH are supposed to be equally reactive, so only the pK_a for $HSRS^-$/HSRSH was taken into account. In these conditions, estimates of k_1 and pK_a are higher than

the real values. Equation 3 can be written in a similar manner to the equation for monothiols Eq. 2:

$$v=\frac{d[\text{Ell-S}^-]}{dt}=k_{1\,obs}([\text{Ell-S-S-Ell}]_0-[\text{Ell-S}^-]_t) \quad (4)$$

$$\text{with}\quad k_{1\,obs}=2k_1\frac{K_a}{K_a+[H_3O^+]}([^-\text{SRS}^-]+[^-\text{SRSH}]+[\text{HSRSH}])_0$$

Equations 2 and 4 can be integrated in:

$$\ln\frac{([\text{Ell-S-S-Ell}]_0-[\text{Ell-S}^-]_t)}{([\text{Ell-S-S-Ell}]_0}=k_{1\,obs}t \quad (5)$$

The $k_{1\,obs}$ values are obtained by plotting the logarithmic expression Eq. 5 *versus* t, and are then plotted *versus* the ratio of total thiol concentration on $[H_3O^+]$ to obtain the product k_1K_a. The reaction of RS^- with Ellman's reagent is well correlated by a Brönsted-type equation, *i.e.* the reactivity is increased as the pK_a of the parent thiol increases ($\log k_1$ is proportional to the pK_a, and the proportionality coefficient is named β_{nuc}). The Brönsted correlation established by Wilson[15] ($\beta_{nuc}=0.49$) enabled us to obtain the k_1 and pK_a values from the product k_1K_a. In the case of trypanothione, although the molecule is not symmetrical, both thiolate functions were considered equivalent. The pK_a obtained in this study does not correspond to the microscopic pK_a value of one given ionized species. It only gives an account of the macroscopic reactivity of $T(SH)_2$.

The k_1 and pK_a values obtained from the first experiments on GSH are $2.0\times10^5\,s^{-1}$ and 8.7, respectively (Table IA). This pK_a is compatible with the value obtained by titration (pK_a 8.83) and the estimate of Bruice (pK_a 8.7).[17] It is 0.4 unit less than the value of pH obtained previously for 50% ionization. The pK_a value obtained for $T(SH)_2$ was 7.4. This value is 0.3 unit less than the pH value obtained for 25% ionization of $T(SH)_2$ in the ionization study. Although GS^- is intrinsically a better nucleophile than $T(S^-)_2$, trypanothione is so ionized that its resulting reactivity is higher. Therefore, the $k_{1\,obs}$ ($k_1[RS^-]_0$), or the reaction rates, are better parameters than k_1 for characterizing the reactivity of a thiol, as both take into account the amount of thiolate form (Table IB).

The observed kinetics of Ellman's reaction are not particular: most reactions of thiolates with electrophiles are well correlated by Brönsted type equations, and β_{nuc} values are generally low (inferior to 0.5—0.7 in aqueous medium).[1] Thus, at pHs around 7, $T(SH)_2$ would react better than GSH on most electrophiles.

Reaction on CDNB The substitution of thiolates to CDNB (Chart 2) was found to be a second order reaction (*SN*2 type). Its kinetic law is analogous to the law obtained in the case of the reaction with Ellman's reagent Eq. 1, and can be written as follows:

$$v=\frac{d[P]}{dt}=k_1[\text{CDNB}][RS^-] \quad (6)$$

with P: thiol–CDNB conjugate

Reaction rates were found to be sufficiently low to consider that the CDNB concentration is constant for 2 min. As previously, we obtained the constant value k_1 [CDNB] by plotting $\ln([RS^-]_0-[P]_t)$ *versus* t. In the case of trypanothione, both thiolate functions were supposed to react in an independent way. Subsequently, the concentration used was the thiol concentration (twice the trypanothione concentration). The ε was measured and found to be equal to $9900\,cm^{-1}\,M^{-1}$ per thiol group ($\varepsilon=9600\,cm^{-1}\,M^{-1}$ for GSH[11]). The similarity between these ε values is compatible with the hypothesis that each thiol of trypanothione reacts independently.

To evaluate the evolution of the rates according to the pH, two kinetic analyses were performed at pH 6.8 and 7.8. This study confirms that the quantity of thiolates is a major factor: when the pH of the solution decreases, the difference between the resulting reactivities of GSH and $T(SH)_2$ increases (Table II). At pH 7.8, the reaction rate is three-fold higher with $T(SH)_2$ than with GSH (9.35 and 3.12 μM min^{-1} respectively). At pH 6.8, it becomes nearly five-fold higher (2.18 and 0.46 μM min^{-1} respectively).

Table I. Rate Constants of the Reaction of GSH and $T(SH)_2$ with Ellman's Reagent and Their Respective pK_as.

A

	pK_a	k_1 ($M^{-1}\,s^{-1}$)
GSH	8.7	2.0×10^5
$T(SH)_2$	7.4	3.5×10^4

B

$[SH]_{total}$ (μM)[a]	pH	k_{1obs} (s^{-1})	
		GSH	$T(SH)_2$
250	4.7	4.4×10^{-3}	18.7×10^{-3}
500	4.7	7.1×10^{-3}	44×10^{-3}

Ellman's reaction was studied according to Wilson's protocol: 0.04 M sodium acetate buffer, pH 4.7, 1 M KCl, 40 μM Ellman's reagent. The appearance of the chromophore was followed at 412 nm and 28 °C. *a*) [SH] = 2[T(SH)$_2$] or [GSH].

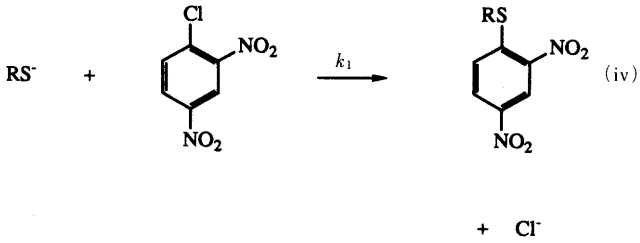


Chart 2. Reaction with 1-Chloro-2,4-dinitrobenzene

Table II. Reaction Rates of the Addition of GSH and $T(SH)_2$ on CDNB

	GSH	$T(SH)_2$	$\frac{v_{GSH}}{v_{T(SH)_2}}$
v[a] (pH 7.8)	3.12	9.35	3.00
v[a] (pH 6.8)	0.46	2.18	4.74
k_1 ($M^{-1}\,s^{-1}$)	2.4±0.2	0.9±0.1	

The reaction was studied in a 0.1 M phosphate buffer, $[SH]_0=1$ mM (*i.e.*, 2[T(SH)$_2$] or [GSH]) and [CDNB] = 250 μM. The reaction rates were determined by following the appearance of the CDNB-thiol conjugate at 340 nm and 28 °C. *a*) v (μM min^{-1}).

Discussion

For numerous organisms, low molecular weight thiols (particularly glutathione or in the case of kinetoplastidae, trypanothione) are key molecules for the physiological defense against oxidative stress and xenobiotic detoxication. Their overriding feature is the presence of the thiol group and its inherent reactivity.

We studied the non-enzymatic reaction of GSH and $T(SH)_2$ on two different electrophilic centres: Ellman's reagent and CDNB. The results obtained are valid for pHs between 6 and 9.5, where the percentage of ionized thiol is higher for $T(SH)_2$ than for GSH. This pH range likely includes the pH of the compartments of *T. cruzi* in which such conjugation reactions are expected to happen. In both cases, around physiological pH and at 28 °C (the temperature of proliferation for epimastigote forms), we observed that the formation of trypanothione S-conjugates is faster than the formation of glutathione S-conjugates. With electrophiles like CDNB, when pH decreases until 5.5, $T(SH)_2$ still reacts when GSH is almost no longer reactive. Although GSH is intrinsically a better nucleophile than $T(SH)_2$, the better percentage of ionization of $T(SH)_2$ at physiological pH makes it more reactive. Spectrophotometric studies[18] have recently shown that GSH is activated by deprotonation when it is complexed with GST. The high amount of $T(SH)_2$ present in trypanosomes (1 to 2 mM-estimated from Fairlamb *et al.*[6]) makes its non-enzymatic conjugation reaction extremely effective with many electrophiles. This might be an explanation for the low activity of GST[9,19] or the absence of activity[20] detected in fresh lysates of *T. cruzi*: the non-enzymatic formation of trypanothione S-conjugates might be efficient enough to eliminate xenobiotics. As for the existence of a trypanothione S-transferase, some activity has been detected in some trypanosomatidae (*Crithidia fasciculata*, *T. brucei brucei*) and in *Leishmania donovani*; nevertheless, no such activity has been found in *T. cruzi*.[21]

In the case of the reaction with Ellman's reagent, the dithiol structure of $T(SH)_2$ results in a kinetic mechanism different from that obtained with GSH. The last equilibrium which leads to the release of $T(S)_2$ and a thiolate anion becomes intramolecular, and consequently much faster than in the case of GSH (Chart 1). In dilute media, which may occur in some organelles, such a structure would be more efficient than GSH to reduce RSSR compounds in their corresponding thiols. Moreover, the presence of a protonated amine in the spermidine bridge could eventually assist in the release of the thiolate anion.[1]

Recent works enlightened other non-enzymatic biological roles of trypanothione. Carnieri *et al.*[8] showed that the peroxidase activity observed in *T. cruzi*[22] was due to non-enzymatic reactions of endogeneous reduced thiols (in particular $T(SH)_2$) with peroxides. Also, Awad *et al.*[23] found that $T(SH)_2$ played an important role in protecting DNA against irradiation by $OH^{\cdot}$ scavenging and H atom donation for chemical repair or restitution processes.

The presence of trypanothione in trypanosomes could have been selected to improve many biological pathways based on glutathione in other species. In all protective means reported here, the non-enzymatic trypanothione based system seems more efficient than the glutathione one. Thus, in trypanosomes the xenobiotic detoxication and the system of defense against oxidative stress would be highly dependent on the amount of $T(SH)_2$ present in the cell. This makes the enzymes involved in the trypanothione metabolism (trypanothione reductase, glutathionyl spermidine synthase, trypanothione synthase[24]) good targets for a specific chemotherapy against trypanosomiosis.

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References and Notes

1) K. T. Douglas, "Glutathione Conjugation, Mechanisms and Biological Significance," ed. by H. Sies, B. Ketterer, Academic Press, Orlando, 1988, pp. 1—41.
2) M. Sonderström, S. Hammarström, B. Mannervik, *Biochem. J.*, **250**, 713 (1988).
3) T. Ishikawa, *TIBS*, **17**, 463 (1992).
4) E. Martinoia, E. Grill, R. Tommasini, K. Kreuz, N. Amrhein, *Nature* (London), **364**, 247 (1993).
5) A. T. Baillie, J. G. Slatter, *Acc. Chem. Res.*, **24**, 264 (1991).
6) A. H. Fairlamb, A. Cerami, *Anu. Rev. Microbiol.*, **46**, 695 (1992).
7) H. Shim, A. H. Fairlamb, *J. Gen. Microbiol.*, **134**, 807 (1988).
8) E. G. S. Carnieri, S. N. J. Moreno, R. Docampo, *Mol. Biochem. Parasitol.*, **61**, 79 (1993).
9) A. Yawetz, M. Agosin, *Comp. Biochem. Physiol.*, **68B**, 237 (1981).
10) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
11) W. H. Habig, M. J. Pabst, W. B. Jakoby, *J. Biol. Chem.*, **249**, 7130 (1974).
12) V. Fauchet, L. Bourel, A. Tartar, C. Sergheraert *Bioorg. Med. Chem. Lett.*, in press.
13) A. El-Waer, K. T. Douglas, K. Smith, A. H. Fairlamb, *Anal. Biochem.*, **198**, 212 (1991).
14) R. E. Benesch, R, Benesch, *J. Am. Chem. Soc.*, **77**, 5877 (1955).
15) J. M. Wilson, R. J. Bayer, D. J. Hupe, *J. Am. Chem. Soc.*, **99**, 7922 (1977).
16) G. M. Whitesides, J. Lilburn, R. P. Szajewski, *J. Org. Chem.*, **42**, 332 (1977).
17) D. M. E. Reuben, T. C. Bruice, *J. Am. Chem. Soc.*, **98**, 114 (1976).
18) A. Karshikoff, P. Reinemer, R. Huber, R. Ladenstein, *Eur. J. Biochem.*, **215**, 663 (1993).
19) C. Moncada, Y. Repetto, J. Aldunate, M. E. Letelier, A. Morello, *Comp. Biochem. Physiol.*, **94C**, 87 (1989).
20) *a*) B. Plumas-Marty, C. Verwaede, M. Loyens, P. Velge, A. Taibi, M.-F. Cesbron, A. Capron, M. A. Ouaissi, *Parasitol.*, **104**, 1 (1992); *b*) This study, data not shown.
21) E. A. O. Etah, K. Smith, A. H. Fairlamb, Abstract of Spring Meeting of the British Society for Parasitology, London, 1993.
22) G. B. Henderson, A. H. Fairlamb, A. Cerami, *Mol. Biochem. Parasitol.*, **24**, 39 (1987).
23) S. Awad, G. B. Henderson, A. Cerami, K. D. Held, *Int. J. Radiat. Biol.*, **62**, 401 (1992).
24) K. Smith, K. Nadeau, M. Bradley, C. Walsh, A. H. Fairlamb, *Protein Sci.*, **1**, 874 (1992).