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# Trypanosomatid hydrogen peroxidase metabolism

P.G. Penketh, W.P.K. Kennedy*, C.L. Patton and A.C. Sartorelli

*MacArthur Center for Parasitology and Tropical Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA* and **Wolfson Molecular Biology Unit, London School of Hygiene and Tropical Medicine, Keppel Street, London WCLE 7HT, England*

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The rate of whole cell $H_2O_2$ metabolism in several salivarian and stercorarian trypanosomes and *Leishmania* species was measured. These cells metabolized $H_2O_2$ at rates between 2.3 and 48.2 nmol/$10^8$ cells per min depending upon the species employed. $H_2O_2$ metabolism was largely insensitive to $NaN_3$, implying that typical catalase and peroxidase haemoproteins are not important in $H_2O_2$ metabolism. The metabolism of $H_2O_2$, however, was almost completely inhibited by *N*-ethylmaleimide. In representative species, $H_2O_2$ metabolism was shown to occur through a trypanothione-dependent mechanism.

Hydrogen peroxide; Trypanothione; Macrophage; (*Trypanosoma, Leishmania*)

## 1. INTRODUCTION

Many trypanosomatids have been reported to lack or to be extremely deficient in enzyme systems necessary for the removal of $H_2O_2$ (i.e. catalase, glutathione peroxidase) [1–5]. Defense mechanisms against $H_2O_2$, however, appear to be a ubiquitous requirement of most aerobic cells [6]. *Leishmania* and *Trypanosoma cruzi* trypomastigotes must also withstand the oxidative killing mechanism of phagocytic macrophages and other host cells [7]. This suggests the presence of mechanisms other than catalase and glutathione peroxidase activities to fulfill this vital role. It has been reported that *L. donovani* possesses a surface-membrane acid phosphatase which reduces the magnitude of the oxidative burst of neutrophils, inferring a pathophysiological role for this enzyme [5]. However, defense mechanisms against endogenously generated $H_2O_2$ and the possibly diminished quantity of $H_2O_2$ produced during the oxidative burst would still be required.

Recently, *T. brucei* has been shown to contain a novel enzymic $H_2O_2$-metabolizing system. This system utilizes NADPH for the reduction of $H_2O_2$ and has an absolute requirement for the dithiol cofactor trypanothione [$N^1$,$N^8$-bis(L-$\gamma$-glutamyl-L-hemicysteinylglycyl)spermidine] [9,10]. Similar activity was measured when organic hydroperoxides were used as substrates, implying that an additional role exists for this activity in the reduction of lipid hydroperoxides [10]. The precise details of the reaction mechanisms involved are not yet clear. A system analogous to the host glutathione reductase/glutathione peroxidase system utilizing trypanothione was anticipated.

Trypanothione reductase has been purified to homogeneity from several trypanosomatides [11,12]. Trypanothione peroxidase activity has been inferred in crude extracts or crude extracts

Correspondence address: P.G. Penketh, MacArthur Center for Parasitology and Tropical Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA

*Abbreviations:* BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; GSSG/GSH, oxidized and reduced glutathione, respectively; NADH, reduced nicotinamide adenine dinucleotide; $NADP^+$/NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate, respectively; $TS_2$/$T(SH)_2$, oxidized and reduced trypanothione, respectively

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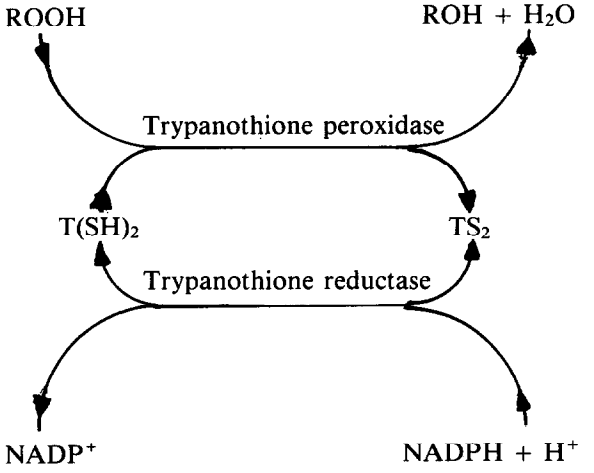
supplemented with additional trypanothione reductase by a presumptive coupled enzymic assay [9,10,13]; however, the expected trypanothione peroxidase activity has so far eluted detection by direct assay in both *T. brucei* and *T. cruzi* [9,10,14]. The following reaction scheme has been proposed for the metabolism of hydroperoxides [13].




Here, we have measured the ability of 12 trypanosomatids to metabolize $H_2O_2$.

## 2. MATERIALS AND METHODS

Enzymes and co-factors were obtained from Sigma. Synthetic trypanothione was a kind gift from Dr A. Fairlamb. All other chemicals were of AR grade. Incubation buffer was composed as in [10].

Bloodstream trypomastigotes of *T. brucei rhodesiense* (YTat 1.1), *T. equiperdum, T. evansi* and *T. vivax* were prepared and purified as described by Lanham [15]. *T. lewisi* and *T. congolense* were prepared in a similar manner, except that lethally irradiated rats were used as hosts. Procyclic forms of *T. brucei rhodesiense* were cultured in Cunningham's medium supplemented with 10% fetal calf serum at 27°C. *T. cruzi* Y strain and X 10 strain epimastigotes were cultured as described by Widmer [16]. *T. cruzi* trypomastigotes (Y strain) were prepared according to Piras et al. [17]. *L. donovani* promastigotes were cultured and isolated as described by Neal [18] for *L. tropica major*. *L. donovani* amastigotes were obtained and purified from infected Syrian hamster spleens as described by Channon et al. [19]. *L. braziliensis* and *L. mexicana amazonensis* promastigotes were cultured in Schneider's medium containing 2 mM glutamine, 10% fetal calf serum, 100 U/ml of penicillin and 100 $\mu$g/ml of streptomycin at 25°C. *L. braziliensis* amastigotes were grown in culture as described by Pan [20]. *L. tarantolae* was cultured in LT medium at 27°C. All cells were washed in incubation buffer (5 mM KCl, 80 mM NaCl, 2 mM $MgCl_2$, 16.2 mM $Na_2HPO_4$, 3.8 mM $NaH_2PO_4$, 50 mM D-glucose, 0.27 mM phenol red and 1.5 mg/ml of BSA, adjusted to pH 7.4) and stored on ice prior to use.

$H_2O_2$ metabolism of cell suspensions was measured as described in the legend to table 1. NADPH dependent $H_2O_2$ consumption was measured as follows. Trypanosomes were washed in glucose-free incubation buffer, incubated for 10 min at room temperature, pelleted by centrifugation (3000 × *g* for 10 min) and osmotically lysed by suspension in distilled water at a density equivalent to $10^9$ cells/ml. This suspension was centrifuged at 20000 × *g* for 10 min and 1 vol. of 1 M Tris-HCl (pH 8.0) containing 10 mM EDTA buffer was added to 9 vols of the supernatant. NADPH and $NADP^+$ were added to give final concentrations of 100 $\mu$M (the inclusion of 100 $\mu$M $NADP^+$ corrects for any residual $NADP^+$ reducing capacity). $H_2O_2$ was then added to the sample cuvette at concentrations between 2.5 and 20 $\mu$M and the change in absorbance at 340 nm was followed at 37°C against an equivalent sample without $H_2O_2$. This procedure was further modified in the case of *T. lewisi* because of the high $NADP^+$ reducing capacity of these lysates. These cells were lysed at a level of $5 \times 10^8$ cells/ml and 250 $\mu$M $NADP^+$ was used instead of 100 $\mu$M NADPH and 100 $\mu$M $NADP^+$. $H_2O_2$ was then added after the mixture had been incubated for 5 min. *L. mexicana amazonensis* promastigotes were treated in a manner analogous to that of *T. lewisi* except that these cells, due to their resistance to osmotic lysis, were lysed at a level of $10^9$ cells/ml by nitrogen cavitation in distilled water. In all of these preparations, the intrinsic trypanothione reductase activity greatly exceeded that of the peroxidase; for example, a 50-fold difference in activity has been reported in *T. brucei* [13]. The $H_2O_2$-mediated oxidation of NADPH

Table 1

The initial rate of $H_2O_2$ metabolism by various trypanosome and *Leishmania* species

| Organism | nmol $H_2O_2$ consumed per $10^8$ cells during the 1st min of exposure to 20 $\mu$M $H_2O_2$ (± SD) |
|---|---|
| Salivarian trypanosomes | |
| *T. brucei rhodesiense* trypomastigotes | 3.5 ± 0.6 (5) |
| *T. brucei rhodesiense* procyclic | 6.4 ± 1.2 (5) |
| *T. equiperdum* trypomastigotes Lump 1559 | 3.7 ± 0.5 (4) |
| *T. evansi* SN trypomastigotes Lump 1559 | 3.5 ± 0.3 (5) |
| *T. evansi* SAK trypomastigotes | 4.2 ± 0.3 (5) |
| *T. vivax* trypomastigotes | 3.5 ± 0.2 (3) |
| *T. congolense* trypomastigotes TREU 1290 | 3.6 ± 0.4 (3) |
| Storcorarian trypanosomes | |
| *T. lewisi* trypomastigotes | 19.6 ± 3.0 (7) |
| *T. cruzi* trypomastigotes Y strain | 48.2 ± 1.9 (3) |
| *T. cruzi* epimastigotes Y strain | 18.3 ± 1.5 (3) |
| *T. cruzi* epimastigotes X 10 strain | 4.2 ± 0.2 (3) |
| *Leishmania* | |
| *L. donovani* promastigotes | 4.3 ± 0.2 (3) |
| *L. donovani* amastigotes | 2.3 ± 0.2 (3) |
| *L. brasiliensis* promastigotes | 8.3 ± 0.3 (3) |
| *L. brasiliensis* amastigotes | 2.3 ± 0.4 (3) |
| *L. mexicana amazonensis* promastigotes | 5.6 ± 0.7 (3) |
| *L. tarentolae* (culture forms) | 3.3 ± 1.1 (4) |

Freshly washed cells were suspended at concentrations of $10^7$–$10^8$ cells/ml, depending upon the species, to obtain conveniently measurable rates in incubation buffer at 37°C. $H_2O_2$ was added to give a final concentration of 20 $\mu$M and 1.2-ml aliquots were removed at 1-min intervals and assayed for residual $H_2O_2$ as described [9]. The values in parentheses indicate the number of samples assayed

was independent of the NADPH concentration within the tested range (i.e. 50–200 $\mu$M).

Ultrafiltrates and high molecular mass fractions were prepared using an Amicon PM-10 filter (nominal molecular mass cut off at 10 kDa). The high molecular mass fraction was washed with 2-times the initial lysate volume of distilled $H_2O$ and suspended in a small volume of $H_2O$. Catalase activity was measured using an $O_2$ electrode as in [9].

## 3. RESULTS AND DISCUSSION

All of the cell lines studied were able to metabolize $H_2O_2$ (table 1). The high consumption of $H_2O_2$ observed in *T. cruzi* trypomastigotes compared to epimastigote forms is consistent with their greater resistance to the phagocytic killing mechanism and to oxidant stress in the form of $H_2O_2$ [21]. The various forms of *L. donovani, L. mexicana, L. braziliensis, T. cruzi* and *T. lewisi* studied showed a progressive decrease in the rate of $H_2O_2$ consumption at relatively low (< 10 $\mu$M) concentrations of $H_2O_2$, whereas the salivarian trypanosomes gave more linear kinetics (not shown). $H_2O_2$ metabolism was largely insensitive to 1 mM $NaN_3$, except for *L. donovani* amastigotes, which implied that typical catalase/peroxidase haemoproteins are not important in the metabolism of $H_2O_2$. $H_2O_2$ metabolism, however, was inhibited 85–100% by pretreatment with NEM (table 2). *L. donovani* amastigotes behaved differently, in that a 40% inhibition of the initial rate of $H_2O_2$ metabolism was produced by 1 mM $NaN_3$, suggesting the presence of a catalase-type activity. An $NaN_3$-sensitive catalase activity was confirmed in *L. donovani* amastigote osmotic lysates using an $O_2$ electrode. This catalase showed an identical electrophoretic mobility to that of Syrian hamster spleen catalase when localized on starch gels (Kennedy, W.P.K., unpublished), suggesting contamination by host enzyme. Catalase is a very 'sticky' enzyme and tends to become attached to the outside of organelles during tissue homogenization. This has led to reports that mitochondria and chloroplasts contained catalase activity. Careful experimentation, however, confirmed the absence of catalase inside these organelles [22,23]. A similar phenomenon may be occurring during the preparation of amastigotes. In support of this premise, no catalase activity was found in in vitro cultured amastigotes and promastigotes of *L. braziliensis*.

Table 2

The effects of the presence of $NaN_3$ and pretreatment with NEM on the initial rate of $H_2O_2$ metabolism by various trypanosome and *Leishmania* species

| Organism | Relative activity in the presence of $NaN_3$ | Relative activity after NEM pretreatment |
|---|---|---|
| *T. brucei rhodesiense* trypomastigotes | 1.03 | 0.02 |
| *T. equiperdum* trypomastigotes Lump 1559 | 0.97 | 0.00 |
| *T. evansi* SN trypomastigotes | 0.97 | 0.00 |
| *T. evansi* SAK trypomastigotes | 1.00 | 0.03 |
| *T. vivax* trypomastigotes | 0.86 | 0.03 |
| *T. cruzi* epimastigotes X 10 strain | 1.05 | 0.15 |
| *T. lewisi* trypomastigotes | 0.89 | 0.00 |
| *L. donovani* promastigotes | 1.02 | 0.07 |
| *L. donovani* amastigotes | 0.61 | 0.39 |
| *L. braziliensis* promastigotes | 0.94 | 0.09 |
| *L. braziliensis* amastigotes | 0.90 | 0.00 |

The initial rate of $H_2O_2$ metabolism was measured as described in the legend to table 1 in the presence of 1 mM $NaN_3$ and also after pretreatment of cells with 0.2 mM NEM at 22°C for 50 min in incubation buffer. Activity is expressed relative to that of untreated controls, which were equated to 1.0

NADPH-dependent $H_2O_2$ consumption was measured in lysates prepared from blood-stream forms of the following species: *T. brucei rhodesiense, T. evansi, T. equiperdum, T. vivax, T. lewisi* and in promastigote forms of *L. mexicana amazonensis*. Addition of $H_2O_2$ in all cases resulted in a stoichiometric and linear (down to about 1 $\mu$M $H_2O_2$) oxidation of NADPH. The stoichiometric consumption of NADPH implies that NADPH-independent $H_2O_2$ consumption is unimportant in these lysates. The linear rate of NADPH oxidation that was observed suggests the presence of an enzymic process with a low $K_m$ value ($< 1$ $\mu$M) for $H_2O_2$ and supports the idea that the rate-limiting diffusion of $H_2O_2$ into cells is the cause of the non-linear kinetics seen with intact cells at low $H_2O_2$ concentrations. This phenomenon would result in intracellular $H_2O_2$ concentrations that are lower than those of the extracellular environment.

Cells lysed at low concentrations showed a disproportionate decrease in activity in this assay, due in part to the presence of subsaturating concentrations of trypanothione. Under the conditions employed, approx. 30% of the intact cellular activity was retained. No activity was observed when NADH was substituted for NADPH.

Large reserves of a compound(s) supporting $NADP^+$ reduction was observed in *L. mexicana* promastigotes and in *T. lewisi*. These reserves could be important in sustaining the defense against prolonged oxidant stress by $H_2O_2$. Very large reserves have also been measured in lysates prepared from *L. donovani* promastigotes and from *T. cruzi* epimastigotes, sufficient to reduce about 30 nmol $NADP^+/10^8$ cells [10].

High molecular mass fractions and ultrafiltrates prepared from blood-stream trypomastigote forms of *T. equiperdum, T. lewisi* and *L. mexicana amazonensis* promastigotes individually showed no $H_2O_2$-dependent NADPH oxidation. However, when the ultrafiltrates and high molecular mass fractions were recombined, activity was restored in a manner approximately proportional to the concentration of the high molecular mass fraction. Addition of GSH (100 $\mu$M) and GSSG reductase (0.1 U/ml) to the high molecular mass fraction did not restore activity, indicating that a GSH peroxidase system was not operative. The addition of concentrated *T. brucei* ultrafiltrate or synthetic trypanothione restored activity in a concentration dependent saturable manner, with 1/2 $V_{max}$ being achieved between 1 and 10 $\mu$M trypanothione, depending upon the species employed. Ultrafiltrates from the above species restored activity in *T. brucei* high molecular mass fractions. These findings indicate that the novel process responsible for $H_2O_2$ metabolism in *T. brucei* is common to the above species. A low glutathione peroxidase activity has been reported in *L. donovani* by Murray [24]; this contrasts with the findings of Channon and Blackwell [5] who indicated that this enzyme was absent. However, the assay used by Murray contained some endogenous trypanothione which may have accounted for the small activity that was observed. The importance and probable general occurrence of this novel trypanothione-dependent process in many if not all

trypanosomatids make it an ideal target for a directed chemotherapeutic attack.

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## REFERENCES

[1] Docampo, R. and Moreno, S.N.J. (1984) in: Free Radicals in Biology, pp.243–288, Academic Press, New York.
[2] Boveris, A., Seis, H., Martino, E.E., Docampo, R., Turrens, J.F. and Stoppani, H.O.M. (1980) J. Biochem. 188, 643–648.
[3] Clark, I.A., Hunt, N.H. and Cowden, W.B. (1986) Adv. Parasitol. 25, 1–28.
[4] Channon, J.Y. and Blackwell, J.M. (1985) Parasitology 91, 197–200.
[5] Channon, J.Y. and Blackwell, J.M. (1985) Parasitology 91, 207–217.
[6] Halliwell, B. (1981) in: Free Radicals, Oxygen Toxicity and Aging, pp.1–62, Elsevier, Amsterdam, New York.
[7] Nguyen, L.T., Meshnick, S.R., Kitchener, K., Eaton, J.W. and Cerami, A. (1983) J. Biol. Chem. 258, 125–130.
[8] Remaley, A.T., Glew, R.H., Huhns, D.B., Basfora, R.E., Waggoner, A.S., Ernst, L.A. and Pope, M. (1985) Exp. Parasitol. 60, 333–341.
[9] Penketh, P.G. and Klein, R.A. (1986) Mol. Biochem. Parasitol. 20, 111–121.
[10] Penketh, P.G. (1986) in: Nitrofurazone and Hydrogen Peroxide Metabolism in *Trypanosoma brucei*, PhD Thesis, Cambridge University.
[11] Krauth-Siegel, R.L., Enders, B., Henderson, G.B., Fairlamb, A.H. and Schirmer, R.H. (1987) Eur. J. Biochem. 164, 123–128.
[12] Shames, S.L., Fairlamb, A.H., Cerami, A. and Walsh, T. (1986) Biochemistry 25, 3519–3526.
[13] Henderson, G.B., Fairlamb, A.H. and Cerami, A. (1987) Mol. Biochem. Parasitol. 24, 39–45.
[14] Schirmer, R.H., Schollhammer, T., Eisenbrand, G. and Krauth-Siegel, R.L. (1987) Free Radical Res. Commun. 3, 3–12.
[15] Lanham, S.M. (1968) Nature 218, 1273–1274.
[16] Widmer, G. (1986) in: A Biochemical and Epidemiological Study of Enzyme Polymorphism in *Trypanosoma cruzi*, PhD Thesis, London School of Hygiene and Tropical Medicine.
[17] Piras, M.M., Piras, R. and Henriques, D. (1982) Mol. Biochem. Parasitol. 6, 67–81.
[18] Neal, R.H. (1984) Exp. Parasitol. 57, 269–273.
[19] Channon, J.Y., Roberts, M.V. and Blackwell, J.M. (1984) Immunology 52, 345–355.
[20] Pan, A.A. (1984) Exp. Parasitol. 58, 72–80.
[21] Tanaka, Y., Tanowitz, H. and Bloom, B.R. (1983) Infect. Immun. 41, 1322–1331.
[22] Day, D.A., Arron, G.P. and Laties, G.G. (1979) J. Exp. Biol. 30, 539–549.
[23] Allen, J.F. (1977) FEBS Lett. 84, 221–224.
[24] Murray, H.W. (1982) J. Immunol. 129, 351–357.