Effect of \( \beta \)-Lapachone on Superoxide Anion and Hydrogen Peroxide Production in Trypanosoma cruzi

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(Received 27 February 1978)

Addition of \( \beta \)-lapachone, an \( o \)-naphthoquinone endowed with trypanocidal properties to respiring *Trypanosoma cruzi* epimastigotes induced the release of \( O_2^- \) and \( H_2O_2 \) from the whole cells to the suspending medium. The same \( \beta \)-lapachone concentration (4 \( \mu \)M) that released \( H_2O_2 \) at maximal rate completely inhibited *T. cruzi* growth in a liquid medium. The position isomer, \( \alpha \)-lapachone, did not stimulate \( O_2^- \) and \( H_2O_2 \) release, and did not inhibit epimastigote growth. \( \beta \)-Lapachone was able to stimulate \( H_2O_2 \) production by the epimastigote homogenate in the presence of NADH as reductant. The same effect was observed with the mitochondrial fraction supplemented with NADH, where \( \beta \)-lapachone enhanced the generation of \( O_2^- \) and \( H_2O_2 \) 4.5- and 2.5-fold respectively. \( \beta \)-Lapachone also increased \( O_2^- \) and \( H_2O_2 \) production (2.5 and 2-fold respectively) by the microsomal fraction with NADPH as reductant. Cyanide-insensitive NADH and NADPH oxidation by the mitochondrial and microsomal fractions (quinone reductase activity) was stimulated to about the same extent by \( \beta \)-lapachone. \( \alpha \)-Lapachone was unable to increase \( O_2^- \) and \( H_2O_2 \) production and quinone reductase activity of the mitochondrial and microsomal fractions.

\( \beta \)-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione), a lipophilic cytoxic \( o \)-naphthoquinone (Lima et al., 1962; Santana et al., 1968), is also an effective trypanocide, causing severe ultrastructural and metabolic alterations in *Trypanosoma cruzi*, the agent of Chagas’ disease (Docampo et al., 1977; Cruz et al., 1978). \( \beta \)-Lapachone stimulates \( H_2O_2 \) generation and lipoperoxide formation in the culture (epimastigote) form of *T. cruzi* and the quinone-treated epimastigotes show a clear e.p.r. signal of the semiquinone, thus indicating the biological reduction of \( \beta \)-lapachone (Docampo et al., 1978a). Since in biological systems naphthoquinone generates \( O_2^- \) and \( H_2O_2 \) (Misra & Fridovich, 1972b), and \( H_2O_2 \) is toxic for trypanosomatidae (Fulton & Spooner, 1956), we have compared the effect of \( \beta \)-lapachone and its biologically inactive position isomer \( \alpha \)-lapachone (3,4-dihydro-2,2-dimethyl-2H-naphtho[2,3-b]pyran-5,10-dione) on \( O_2^- \) and \( H_2O_2 \) generation and growth of *T. cruzi* (Tulahuen strain) *in vitro*. In the present paper, we report a correlation between the ability of \( \beta \)- and \( \alpha \)-lapachone to produce \( O_2^- \) and \( H_2O_2 \) and their action on the growth of *T. cruzi*.

Despite the fact that Chagas’ disease, caused by *T. cruzi*, is an important health problem in Latin America, a therapeutic agent effective against all forms of the parasite has not yet been found. A rational approach to the design of a trypanocidal drug is the investigation of substances such as \( \beta \)-lapachone, which, by stimulating the production of toxic metabolites, may lead to a lethal situation for the parasite.

Materials and Methods

The Tulahuen strain of *T. cruzi* was grown in a liquid medium consisting of brain–heart infusion (Difco Laboratories, Detroit, MI, U.S.A.) 37g, haemin chlorhydrate (dissolved in triethanolamine) 20mg, and bovine serum, 100ml/litre (Warren, 1960). Epimastigote growth was determined by cell counting in a Neubauer chamber. Quinones in ethanolic solution were aseptically added to the culture medium. The corresponding controls showed no effect of the amount of added ethanol on epimastigote growth. For the preparation of subcellular fractions, the epimastigotes were disrupted by freezing at \(-16^\circ\)C and thawing three times. The cells were suspended in 0.23M-mannitol/0.07M-sucrose/1mM-EDTA/10mM-Tris/HCl, pH 7.2, at 8mg of protein/ml, and were homogenized by several passages through a no. 24-gauge hypodermic needle attached to a syringe. The homogenates were fractionated in a Sorvall RC-2B centrifuge at 2°C. The fractions obtained were: (a) the nuclear–flagellar fraction, which amounted to 20% of the total protein; (b) the mitochondrial fraction, sedimented at 12000g for 10min,
which accounted for 40% of the total protein; (c) the microsomal fraction, sedimented at 105000 g for 45 min, which accounted for 7% of the protein; (d) the supernatant, containing 33% of total protein. The specific activities of succinate dehydrogenase, selected as marker enzyme and determined by the Arrigoni & Singer (1962) assay, were 4.3, 13.6, 5.1 and 0 nmol of succinate/min per mg of protein in fractions (a), (b), (c) and (d) respectively. The protein content of cell suspensions and subcellular fractions was determined by the biuret assay (Gornall et al., 1949) in the presence of 0.2% sodium deoxycholate.

The rates of H₂O₂ generation were determined by measuring the rate of horseradish peroxidase H₂O₂ formation by dual-wavelength spectrophotometry at 417–402 nm (Δε = 50 litre·mmol⁻¹·cm⁻¹; Boveris et al., 1972). The rates of O₂⁻ generation were determined by measuring adrenochrome formation by dual-wavelength spectrophotometry at 485–575 nm (Δε = 2.97 litre·mmol⁻¹·cm⁻¹; Misra & Fridovich, 1972a; Cadenas et al., 1977). The rates of O₂⁻ generation were calculated from the superoxide dismutase-sensitive rate of adrenochrome production, which was usually higher than 95% of the total rate of adrenochrome formation. Quinone reductase activity was measured by following either NADH or NADPH oxidation at 340 nm in a Gilford 2000 spectrophotometer. Mitochondrial and microsomal fractions (0.2–0.5 mg of protein/ml) were suspended in 50 mm-potassium phosphate buffer, pH 7.4, containing 1 mm-KCN, 100 μM-NADH (or NAPD) and quinone (0–40 μM). All enzyme reactions were measured in a thermostatically controlled cell compartment at 30°C. The rates of generation of O₂⁻ and H₂O₂ by quinols in buffered solutions were measured as described above. Quinol concentrations were determined by recording their u.v. absorption spectra (Cadenas et al., 1977) with a Beckman DK-2 spectrophotometer. The second-order reaction constants for the production of O₂⁻ (k₃) and H₂O₂ (k₄) in the autoxidation reaction of quinols were calculated from the linear relationship between the amount of quinol and the rate of O₂⁻ and H₂O₂ generation. Quinols were diluted to 1–30 μM in 50 mm-Tris/Mops (4-morpholinepropanesulfonic acid), pH 7.4, as indicated previously (Cadenas et al., 1977).

**Chemicals**

β- and α-lapachone were provided by Dr. S. Albónico, Department of Pharmaceutical Sciences, School of Pharmacy and Biochemistry, University of Buenos Aires. Quinone concentration and purity were determined by recording their u.v. absorption spectra in a Beckman DK-2 spectrophotometer. β-Lapachone has an ε₂₅₀ of 40 litre·mmol⁻¹·cm⁻¹ and an ε₄₄₀ = 3.1 litre·mmol⁻¹·cm⁻¹ and α-lapachone has an ε₂₅₀ = 31 litre·mmol·cm⁻¹, both in ethanolic solution (Fig. 1). Quinols were prepared by addition of 0.1 ml of a KBH₄ solution (30 mg of KBH₄/ml of 0.1 M-KOH) to 0.8 ml of an approx. 10 mm-quinone solution in dimethylformamide/water (3:7, v/v). HCl (0.1 ml of 0.2 M) was added to decompose excess borohydride, and the final clear solution was supplemented with 10 μl of 0.1 M-EDTA.

NADH, NADPH, adrenaline bitartrate, horseradish peroxidase type VI and bovine superoxide dismutase were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

**Results**

**Observations with intact epimastigotes and homogenates**

Respiring *T. cruzi* epimastigotes did not release either O₂⁻ or H₂O₂ to the suspending medium as detected by the adrenochrome assay for O₂⁻ and by H₂O₂-horseradish peroxidase formation for H₂O₂ (Fig. 2a and 2b). The lack of release of H₂O₂ from whole cells had been previously observed in spite of the active H₂O₂ generation of *T. cruzi* cells (Boveris & Stoppani, 1977), which implies the operation of an intracellular H₂O₂-detoxifying system (Docampo et al., 1976). Addition of β-lapachone initiated an immediate production of adrenochrome, which was sensitive to superoxide dismutase, indicating O₂⁻ generation (Fig. 2b). At low β-lapachone concentrations (up to 4 μM) O₂⁻ and H₂O₂ were released in the ratio of 1 O₂⁻ to 3 H₂O₂ (Fig. 3) and at the higher quinone concentrations, O₂⁻ release kept a linear relationship with quinone concentration. In contrast

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**Fig. 1.** U.v.-absorption spectra of β-lapachone (a) and α-lapachone (b)

--- Oxidized form; ----, reduced form. A 1 cm light-path was used. Other conditions were as described in the Materials and Methods section.
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Fig. 2. Production of \( H_2O_2 \) and \( O_2^- \) in T. cruzi epimastigotes on addition of \( \beta \)-lapachone

(a) Production of \( H_2O_2 \) measured at 417–420 nm. The incubation mixture contained: 154 mM-NaCl, 20 mM-potassium phosphate buffer, pH 7.2, 0.5 \( \mu \)M-horseradish peroxidase (HRP), 0.5 mg of cell protein/ml. The value against the trace indicates \( H_2O_2 \) released/min per \( 10^6 \) cells (in pmol). (b) Production of \( O_2^- \) measured at 485–575 nm. The incubation mixture contained: 154 mM-NaCl, 20 mM-potassium phosphate buffer, pH 7.2, 1 mM-adrenaline, 1.75 mg of cell protein/ml. L, 13 \( \mu \)M-\( \beta \)-lapachone. The value against the trace indicates pmol of \( O_2^- \) released/min per \( 10^6 \) cells. Abbreviations used: L, 5 \( \mu \)M-\( \beta \)-lapachone; SOD, 5 \( \mu \)g of superoxide dismutase/ml.

with these results, \( H_2O_2 \) release reached a plateau and even slightly decreased. The plateau formation could be real and reflect saturation of intracellular superoxide dismutase (Boveris & Stoppani, 1977), a possibility that is compatible with the relatively short diffusional distances between the mitochondrial arms (where much of \( O_2^- \) generation occurs) and the surface of the cell (Paulin, 1975; Meyer & de Souza, 1976; Docampo et al., 1977). However, it could also reflect an interference of \( \beta \)-lapachone-reduced forms with the rate of formation of the horseradish peroxidase–\( H_2O_2 \) complex (the spectrophotometric indicator), since quinols can act as hydrogen donors for the peroxidase reaction (Yamazaki et al., 1960). At variance to these results, \( \alpha \)-lapachone was unable to produce any significant release of \( O_2^- \) and \( H_2O_2 \) from the epimastigotes (Fig. 3).

\( T. cruzi \) homogenates supplemented with reduced nicotinamide nucleotides are effective sources of \( H_2O_2 \) (Boveris & Stoppani, 1977). Addition of \( \beta \)-lapachone increased \( H_2O_2 \) production by these homogenates significantly (about 3-fold the control rate at 30 \( \mu \)M-\( \beta \)-lapachone; Fig. 3, inset). The reason for the declining \( H_2O_2 \) rates at the higher quinone concentration is probably the abovementioned quinol interference with the horseradish peroxidase–\( H_2O_2 \) complex. A similar but lesser stimulation of \( H_2O_2 \) generation by \( \beta \)-lapachone was observed in the presence of NADPH as reductant (Fig. 3, inset). In contrast with the effects of \( \beta \)-lapachone, \( \alpha \)-lapachone did not increase \( H_2O_2 \) production by the homogenate, in good agreement with its lack of effect in stimulating \( O_2^- \) or \( H_2O_2 \) release from epimastigotes (Fig. 3 and Fig. 3, inset). The stimulation by \( \beta \)-lapachone of \( H_2O_2 \) production was about twice that with the homogenate as with the intact epimastigotes. Thus with intact epimastigotes 5 \( \mu \)M-\( \beta \)-lapachone induced the release of 0.5 nmol of \( H_2O_2 \)/min per mg of protein (Fig. 3), whereas with the homogenate the same quinone concentration increased \( H_2O_2 \) production by 1 nmol/min per mg of protein (Fig. 3, inset). This difference is consistent with the existence of an intracellular \( H_2O_2 \)-utilizing system (Docampo et al., 1976). In this connection, it is worth recalling that addition of \( \beta \)-lapachone also increased superoxide production by \( T. cruzi \) amastigote and epimastigote homogenates (Sonya strain) (Docampo et al., 1978c).

Observations with subcellular fractions

Fractionation of \( T. cruzi \) homogenates by differential centrifugation yields fractions that contain fragments derived from the intracellular organelles. Although these fractions are far from possessing the purity of those obtained from mammalian tissues, they are fairly well characterized. Mitochondrial fractions from \( T. cruzi \) show NADH oxidase, NADH dehydrogenase and succinate dehydrogenase activities, and contain a cytochrome system with cytochromes \( a + a_s \), \( b \) and \( c_{558} \) (Boiso & Stoppani, 1971; Agosin et al., 1976; Docampo et al., 1978b). Microsomal fractions show an antimycin-insensitive NADH–cytochrome \( c \) reductase activity and contain cytochrome \( P-450 \) (Boiso & Stoppani, 1971; Agosin et al., 1976).
Fig. 3. Effect of β-lapachone and α-lapachone on $O_2^-$ and $H_2O_2$ release from whole epimastigotes
Experimental conditions were as in Fig. 2. $\blacktriangle$, $O_2^-$ release with β-lapachone; $\bullet$, $H_2O_2$ release with β-lapachone; $\blacktriangledown$, $O_2^-$ release with α-lapachone; $\circ$, $H_2O_2$ release with α-lapachone. The inset shows the effect of β-lapachone and α-lapachone on $H_2O_2$ production by the homogenate of T. cruzi epimastigotes. The incubation mixture contained: 130mM-KCl, 20mM-potassium phosphate buffer, pH 7.2, 40mM-NADH or -NADPH, 0.6μM-horseradish peroxidase, 0.1-0.2mg of homogenate protein/ml.

In good agreement with previous observations (Boveris & Stoppani, 1977), the mitochondrial fraction of T. cruzi actively generated $H_2O_2$ in the presence of NADH (Fig. 4a). Use of reduced nicotinamide nucleotides as electron donors was compulsory since, at variance with other mitochondrial preparations (Boveris & Chance, 1973; Boveris & Cadenas, 1975; Boveris et al., 1976; Boveris, 1977), with T. cruzi mitochondrial fragments, succinate (and antimycin) do not modify the rate of $H_2O_2$ production. Addition of β-lapachone to the mitochondrial fraction supplemented with NADH, stimulated $O_2^-$ production linearly up to 40μM-quinone, at which concentration the basal rate was increased 4.5 times (Fig. 4a). Production of $H_2O_2$ was stimulated about 2.5 times with a maximum at about 30μM-quinone (Fig. 4a). Lesser effects were observed with NADPH as reductant. Addition of α-lapachone did not stimulate either $O_2^-$ or $H_2O_2$ production in the presence of either NADH or NADPH as reductant (results not shown). The effect of β-lapachone on $H_2O_2$ production with the mitochondrial fraction was in accordance with that observed with the homogenate, for NADH was more effective than NADPH as substrate and saturation was observed at about 30μM-quinone.

Production of $O_2^-$ and $H_2O_2$ by the microsomal fraction was also stimulated by β-lapachone (Fig. 4b). The results correspond to our best microsomal preparation, judging by the nicotinamide nucleotide specificity for $O_2^-$ and $H_2O_2$ production. In the presence of NADPH, the quinone was able to stimulate $O_2^-$ and $H_2O_2$ generation by about 2-fold, the maximal stimulation being reached at about 20μM-β-lapachone. Much lesser effects were observed in the presence of NADH. Addition of α-lapachone to
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The effects of NADPH or NADH on the microsomal fraction were determined with NADH or NADPH as electron donors in the presence of cyanide and quinone (Fig. 5). The rate of NADH-oxidation by mitochondrial fragments was stimulated about 3-fold by 40 μM-β-lapachone, whereas NADPH oxidation was less stimulated. It must be noted that α-lapachone did not stimulate NADH or NADPH oxidation by the mitochondrial fragments. Similar effects of β- and α-lapachone were found when the quinone reductase activity of microsomal preparations was determined. In this case, NADPH was more effective than NADH as reductant (Fig. 5, inset). β-Lapachone at 20–25 μM was able to increase the cyanide-insensitive NADPH oxidation by the microsomal fraction about 5-fold, whereas NADH oxidation was less stimulated. α-Lapachone had no effect on the cyanide-insensitive NADH or NADPH oxidation by microsomal fragments.

The rates of nicotinamide nucleotide oxidation by the mitochondrial and microsomal fractions (Fig. 5) were 90–180% higher than the rates of H2O2 production by the same fractions, irrespective of β-lapachone addition (Figs. 4a and 4b). The relatively lower rates of H2O2 production are understandable considering that (a) some reduced nicotinamide nucleotide oxidation did not lead to H2O2 formation and (b) the horseradish peroxidase-H2O2 assay slightly underestimated H2O2 production (Boveris et al., 1977).

The increased rate of O2− and H2O2 production after β-lapachone addition to the T. cruzi preparation involved the autooxidation of the corresponding quinols. Consequently the rate of the autooxidation reactions was measured and is expressed in Table 1 as the second-order reaction constants corresponding to eqns. (1) and (2)

\[
d\left[O_2^−\right]/dt = k_3[LH_2][O_2]
\]

(1)

\[
d[H_2O_2]/dt = k_4[LH_2][O_2]
\]

(2)

(menadiol is included for purposes of comparison). In spite of its more positive \( E_0' \), reduced β-lapachone was much more effective than α-lapachone as H2O2 generator, a characteristic derived from its o-quinone nature. For the other two 6-quinones, namely, α-lapachone and menadiol, the \( k_4 \) values reflect the \( E_0' \) values.

By comparison of the rates of quinol production and oxidation, the redox steady state of the β-lapachone system was calculated. For instance, at 10 μM-quinone and a mitochondrial protein concentration of 0.1 mg/ml, the rate of quinol production was about 0.18 μmol/min per litre (Fig. 5), whereas at 10 μM-quinol, the rate of the oxidation was 11.4 μmol/min per litre (Table 1), which means that in the steady state, the β-lapachone couple would be 1.6% reduced and 98.4% oxidized. This calculation was confirmed (experimental results not given) by measuring the lack of effect of NADH on the bleach-

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Fig. 4. Effect of β-lapachone on O2− and H2O2 production by the mitochondrial and microsomal fractions of T. cruzi

(a) Experiment with the mitochondrial fraction. The incubation mixture contained: 130 mM-KCl, 20 mM-potassium phosphate buffer, pH 7.2, either 0.6 μM-horseradish peroxidase or 1 mM-adrenaline for H2O2 or O2− measurements respectively, 40 μM-NADH or -NADPH, 0.1–0.4 mg of protein/ml. •, H2O2 production with NADH; ○, O2− production with NADH; △, H2O2 production with NADPH, ▲, O2− production with NADPH.

(b) Experiment with the microsomal fraction; the conditions and symbols as for (a).

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the microsomal preparations did not produce any effect on O2− and H2O2 production with either NADPH or NADH as substrate (results not shown).

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Fig. 5. Effect of β-lapachone and α-lapachone on the cyanide-insensitive NADH- and NADPH-oxidase activity of the mitochondrial and microsomal fraction of T. cruzi

The main graph and the inset show the data corresponding to the mitochondrial and microsomal fractions respectively. The incubation mixture contained 50 mM-potassium phosphate buffer, pH 7.2, and 0.1–0.2 mg of protein/ml. The other experimental conditions were as indicated in the Materials and Methods section. •, NADH and β-lapachone; ○, NADH and α-lapachone; △, NADPH and β-lapachone; □, NADPH and α-lapachone.

Table 1. Second-order reaction constants for O₂⁻ and H₂O₂ production in the autoxidation reaction of reduced lapachones

Quinols were prepared as described in the Materials and Methods section, diluted in 50 mM-Tris/Mops pH 7.4 and used in the concentration range of 1–30 μM. The second order reaction of quinols were calculated from the linear relationship between the amount of quinol and the rate of O₂⁻ and H₂O₂ generation, according to eqns. (1) and (2) (see the text). The oxygen concentration was kept constant. Other experimental details were as indicated previously (Cadenas et al., 1977). E₀’ (at pH 7.4) calculated from values given by Fieser & Fieser (1960).

<table>
<thead>
<tr>
<th>Quinol</th>
<th>E₀’ (mV)</th>
<th>k₃ (M⁻¹·s⁻¹)</th>
<th>k₄ (M⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced β-lapachone</td>
<td>-17</td>
<td>0.13</td>
<td>5200</td>
</tr>
<tr>
<td>Reduced α-lapachone</td>
<td>-120</td>
<td>2.78</td>
<td>23</td>
</tr>
<tr>
<td>Menadiol</td>
<td>-60</td>
<td>1.38</td>
<td>5</td>
</tr>
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</table>

Effect on growth

Growth of T. cruzi epimastigotes was brought practically to a standstill by 5 μM-β-lapachone (Fig. 6) since the growth constant k (k = 0.693/T; T = generation time) was decreased by a factor of 20. Conversely, α-lapachone was almost inactive, since 30 μM-quinone only decreased k by about 20%.

Discussion

T. cruzi epimastigotes are effective H₂O₂ generators. According to our data on specific activity of cellular fractions and distribution of subcellular...
Fig. 6. Effect of $\beta$-lapachone and $\alpha$-lapachone on growth of $T. cruzi$ epimastigotes

Experimental conditions were as described in the Materials and Methods section. The inset shows the effect of the quinones on the growth constant, $k$. ●, $\beta$-lapachone; ○, $\alpha$-lapachone.

protein (Boveris & Stoppani, 1977), the epimastigotes would produce, with an adequate substrate supply, about 260nmol of $H_2O_2$/min per g wet weight, which is about three times as high as the $H_2O_2$ production by rat liver cells (90nmol/min per g wet tissue; Boveris et al., 1972). The main defence mechanism of $T. cruzi$ epimastigotes against the accumulation of the intermediates of the partial reduction of oxygen ($O_2^-$ and $H_2O_2$) includes two enzymes, namely, superoxide dismutase and peroxidase. Superoxide dismutase is present in the homogenate with an activity equivalent to 0.28 $\mu$g of bovine superoxide dismutase/mg of protein (Boveris & Stoppani, 1977). The ascorbate-utilizing peroxidase is microperoxisomal, but it is also in the cytosol to a limited extent (Docampo et al., 1976). It must be noted that peroxidase reaction requires one hydrogen donor molecule per $H_2O_2$ molecule utilized and, consequently, the rate of $H_2O_2$ detoxication is determined by the rate of hydrogen donor production. $T. cruzi$ as other members of the tripanosomatidae family, does not contain catalase (Docampo et al., 1976), and diffusion of $H_2O_2$ through cell membrane to the extracellular medium as a defence mechanism does not seem to be operative under physiological conditions (Fig. 2). In consequence, catalase-lacking trypanosomes (like $T. cruzi$) should have intracellular $H_2O_2$ concentrations that far exceed those of mammalian tissues. In this context, Meshnick et al. (1977) have reported 70$\mu$M-$H_2O_2$ in Trypanosoma brucei (an African trypanosome), approximately 30 times as high as in rat liver, namely 2.6$\mu$M (the other report gives $H_2O_2$ in rat liver as 1–100nm; Oshino et al., 1973). Since $H_2O_2$ is toxic for $T. cruzi$ and other members of the Trypanosomatidae (Fulton & Spooner, 1956), substances that increase $H_2O_2$ generation or decrease $H_2O_2$ utilization (Kusel et al., 1973; Boveris & Stoppani, 1977), or that catalyse the homolytic breakdown of $H_2O_2$ (Meshnick et al., 1977) are potential trypanocidal drugs. No direct demonstration of superoxide toxicity towards $T. cruzi$ has been reported. Nevertheless, when ten different $\beta$-lapachone-related naphthoquinones were assayed for superoxide-anion generation and toxic action on $T. cruzi$, the correlation coefficient ($r$) was 0.88 ($P < 0.01$), thus suggesting a toxic action of the superoxide anion (Boveris et al., 1978).

Addition of $\beta$-lapachone to $T. cruzi$ epimastigotes increases the intracellular rate of $O_2^-$ and $H_2O_2$ generation and releases the oxygen reduction intermediates to the suspending medium. The immediate response indicates a rapid permeation of the quinone through the epimastigote cell membrane. The chemical reactions that would explain the trypanocidal action of $\beta$-lapachone (L) could be written:

$$\text{NAD(P)H} + H^+ + L \rightarrow \text{NAD(P)}^+ + LH_2 \quad (3)$$

$$\text{LH}_2 + L \leftrightarrow 2\text{LH}^+ \quad (4)$$

$$\text{LH}_2 + O_2 \overset{k_4}{\rightarrow} L + H_2O_2 \quad (5)$$

$$\text{LH}_2 + O_2 \overset{k_3}{\rightarrow} \text{LH}^- + O_2^- + H^+ \quad (6)$$

$$\text{LH}^- + O_2 \rightarrow L + O_2^- + H^+ \quad (7)$$

$$O_2^- + O_2^- + 2H^+ \leftrightarrow H_2O_2 + O_2 \quad (8)$$

$$O_2^- + H_2O_2 \leftrightarrow O_2 + OH^- + HO^- \quad (9)$$

Reaction (3) is the well known quinone reductase reaction (Crane, 1961; Slater et al., 1961; Brodie, 1965; Ruzicka & Crane, 1971) that takes place in the mitochondrial and endoplasmic-reticulum membranes, where the lipophilic character of $\beta$-lapachone molecules should play an important role. Mitochondrial membranes and NADH are more important by far than the endoplasmic reticulum and NADPH as the system that reduces $\beta$-lapachone in $T. cruzi$, considering (a) the similarity between the effect of $\beta$-lapachone on $H_2O_2$ production by the homogenate and by the mitochondrial fraction, and (b) the protein distribution and the specific activity of the respective subcellular fractions. Quinone reduced forms are enzymically oxidized by molecular oxygen, yielding $H_2O_2$ and $O_2^-$ [reactions (5)–(7)]. With $\beta$-lapachone and $T. cruzi$ subcellular fractions, quinone reduction [reaction (3)] is slower than quinone oxidation [reactions (5)–(7)], $\beta$-lapachone usually being kept in a highly (over 98%) oxidized state.
state. Semiquinone formation [reaction (4)] is a very rapid process in quinol/quinone mixtures (Michaelis, 1951; Yamazaki & Ohnishi, 1966) and could be even more so when the molecules are bound to a membrane. Incidentally, mitochondrial membranes appear to stabilize ubisemiquinone by binding it to a specific site (Cadenas et al., 1977; Ingledew et al., 1976; Salerno et al., 1977). Subcellular fractions and T. cruzi epimastigotes produce and release O$_2^-$ and H$_2$O$_2$ in rather similar amounts, implying the function of reactions (6) and (7). Semiquinone autoxidation [reaction (7)] appears to be the main ratelimiting step of this set of reactions as shown by: (a) the model autoxidation reaction of mitochondrial ubisemiquinone (Cadenas et al., 1977); (b) the fact that O$_2^-$ and H$_2$O$_2$ are produced in similar amounts in the mitochondrial samples, although fully reduced $\beta$-lapachone mainly generates H$_2$O$_2$; (c) the detection of the semiquinone free radical in T. cruzi epimastigotes treated with $\beta$-lapachone (Docampo et al., 1978a). Superoxide anion dismutation yields H$_2$O$_2$ [reaction (8)]. The Haber–Weiss reaction [reaction (9)] generating either hydroxyl radical (Fong et al., 1973; Zimmerman et al., 1973) or singlet oxygen (Kellog & Fridovich, 1975) seems to be the most reasonable mechanism underlying the complex chain reaction that leads to extensive lipid and organic peroxide formation and to biological damage. In this connection it seems pertinent to recall that T. cruzi epimastigotes supplemented with $\beta$-lapachone show increased lipid peroxidation (Docampo et al., 1978a). The metabolic and ultrastructural alterations observed in $\beta$-lapachone-treated T. cruzi cells (Docampo et al., 1977; Cruz et al., 1978) could be easily accounted for by extensive organic and lipid peroxide formation, leading to enzyme inactivation and membrane damage.

Another amphipathic naphthoquinone, vitamin K$_1$, has been reported to act as electron carrier in artificial bulk membranes (Anderson et al., 1976), an experimental fact that supports the hypothesis that quinones may interfere in proton conductance in the mitochondrial membranes and, consequently, uncouple oxidative phosphorylation, especially in the ubiquinone–cytochrome b region (Mitchell, 1975a,b). $\beta$-Lapachone does indeed uncouple oxidative phosphorylation in rat liver mitochondria; however, the effective uncoupling concentration (20 $\mu$m; Docampo et al., 1977) is about five times as high as that (4 $\mu$m) which produces maximal stimulation of H$_2$O$_2$ release (Fig. 3) and completely inhibits T. cruzi growth (Fig. 6). Ultrastructural alterations in $\beta$-lapachone-treated T. cruzi cells were also different to those observed in epimastigotes treated with other uncoupling agents such as 2,4-dinitrophenol (Docampo, 1977). So, the uncoupling activity of $\beta$-lapachone can be excluded as an explanation of the present results.

It is noteworthy, that other bactericidal and cytotoxic drugs, such as streptonigrin (Gregory & Fridovich, 1973), adriamycin (Thayer, 1977), toxoflavin (Latusasan & Berends, 1961), mitomycin C (Tomasz, 1976) etc., probably share with $\beta$-lapachone the same general mechanism of biological action. Specifically, with respect to trypanosomatidae, naphthoquinones, by increasing the rate of H$_2$O$_2$ generation and haematoporphyrin (Meshnick et al., 1977) by increasing hydroxyl radical formation, provide molecular models that could develop into practical trypanocidal drugs.

This investigation was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Secretary of State of Science and Technology (Program of Chagas' Disease), Argentina, and the Scientific Office of the Organization of American States. R. D. was a grantee of the World Health Organization. A. B., R. D. and A. O. M. S. are Career Investigators from CONICET.

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EFFECT OF β-LAPACHONE ON PEROXIDE PRODUCTION IN *T. CRUZI*


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