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Generation of Superoxide Anions and Hydrogen Peroxide from β-Lapachone in Bacteria

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β-Lapachone markedly increased the generation of superoxide anions and hydrogen peroxide by subcellular membranes of *Bacillus subtilis* and *Bacillus stearothermophilus*. Peroxide generation by β-lapachone was parallel to the inhibition of growth in both microorganisms.

Intracellular reduction followed by autoxidation, yielding O_2^- and H_2O_2, has been suggested as the mode of action of several antibiotics and other inhibitory agents. Thus, toxoflavin (14), streptonigrin (10), 4-nitroquinoline *N*-oxide (2), various nitrofurans (2) and quinones (22), Na_2PtCl_6 (17), Adriamycin (doxorubicin) (1, 8, 12, 16, 20), and mitomycin C (21) were shown to act in various microorganisms and cell extracts as electron carriers between reduced nicotinamide adenine dinucleotide (NADH) (or reduced NAD phosphate) and oxygen, with concomitant production of either the superoxide anion or hydrogen peroxide.

β-Lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphtho [1,2-b]pyran-5,6-dione), an antimicrobial (15) and antitumor (18) *o*-naphthoquinone, has been shown to possess similar O_2^-- and H_2O_2-generating properties in mitochondrial and microsomal suspensions as well as in intact cells of *Trypanosoma cruzi* (5, 6) and sarcoma 180 ascites tumor cells (R. Docampo, F. S. Cruz, A. Boveris, R. P. A. Muñiz, and D. M. S. Esquivel, Biochem. Pharmacol., in press).

In this communication we describe evidence which supports the proposal that the toxicity of this quinone is expressed through O_2^- and H_2O_2 formation. It is also shown that a close 2:1 stoichiometry exists between O_2^- and H_2O_2 production by subcellular membranes of *Bacillus subtilis* and *Bacillus stearothermophilus*.

B. stearothermophilus strain 1503-4R was obtained from J. Cannata. The cells were grown in a liquid medium composed of Casitone (Difco, 12 g), yeast extract (Difco, 1 g), NaCl (8 g), $CaCl_2$ (0.19 g) and water (1 liter) or in a solid medium composed of meat extract (Corporación Argentina de Productores de Carme, Argentina, 3 g), peptone (Difco, 10 g), yeast extract (Difco, 3 g), agar (Difco, 30 g), and water (1 liter). *B. subtilis* cells were grown in a medium composed of peptone (Difco, 10 g), beef extract (Difco, 3 g), NaCl (5 g), sucrose (2 g), and water (1 liter) or in the solid medium described above. Cultures were grown at 60°C (*B. stearothermophilus*) and 37°C (*B. subtilis*) and were monitored turbidimetrically at 600 nm (10). Cells were harvested and washed twice in saline. Bacterial membranes were prepared by ultrasonic fragmentation of cells suspended in 0.3 M sucrose–5 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.3)–1 mM ethylenediaminetetraacetate at 0°C, for 1 min with an MSE ultrasonic disintegrator (Measuring and Scientific Equipment, London) operated at a power output of 90 W, followed by centrifugation for 15 min at 10,000 × *g* and 60 min at 105,000 × *g*. Protein was determined by the biuret method (9).

The growth-inhibiting concentrations indicated in Table 1 were found to depend on the culture medium and methods used, so the numbers give only a comparative value. However, it was consistently found that the growth of *B. subtilis* was at least two times more sensitive to β-lapachone than that of *B. stearothermophilus*.

To show that there was an oxygen dependence to the β-lapachone effect, *B. stearothermophilus* and *B. subtilis* were exposed to β-lapachone under aerobic or stringently anaerobic conditions. Anaerobic conditions were achieved by sweeping the cell suspensions with pure N_2 for 1 h before the addition of 40 μM β-lapachone. The cells were then incubated at 60°C (*B. stearothermophilus*) or 37°C (*B. subtilis*), and at 30 min samples were removed, diluted, and plated onto nutrient agar plates, which were incubated for 24 h at 60 or at 37°C, respectively, for quantitation of surviving cells. Controls were performed in which β-lapachone was not added. There was 85 to 90% viability of cells among those exposed to β-lapachone under anaerobic conditions and only 2 to 4% viability among cells exposed to β-lapachone under aerobic conditions.

To have a quantitative relationship between the rate of superoxide radical and hydrogen peroxide formation in bacterial membranes, the adrenochrome assay (5) was used, and H_2O_2 was measured by the formation of horseradish peroxidase-H_2O_2 complex II (4).

The rates of O_2^- production in *B. stearothermophilus* and *B. subtilis* subcellular fragments, as detected by the adrenochrome assay, are illustrated in Fig. 1A and C, respectively. Upon addition of 20 μM β-lapachone no significant O_2^- production was observed. Further addition of NADH caused a 2.5-times stimulation of O_2^- production as compared with the preparation without β-lapachone. This O_2^- production was specifically inhibited by superoxide dismutase

TABLE 1. *Growth inhibition of B. subtilis* and *B. stearothermophilus* by β-lapachone[a]

Microorganism	Concn of β-lapachone causing 50% inhibition of growth (μM)
B. subtilis	10
B. stearothermophilus	18

[a] Cells were grown in the media described in the text during 24 h at 60°C (*B. stearothermophilus*) or 37°C (*B. subtilis*). β-Lapachone was added aseptically as an ethanolic solution (the amount of ethanol added had no effect on growth).

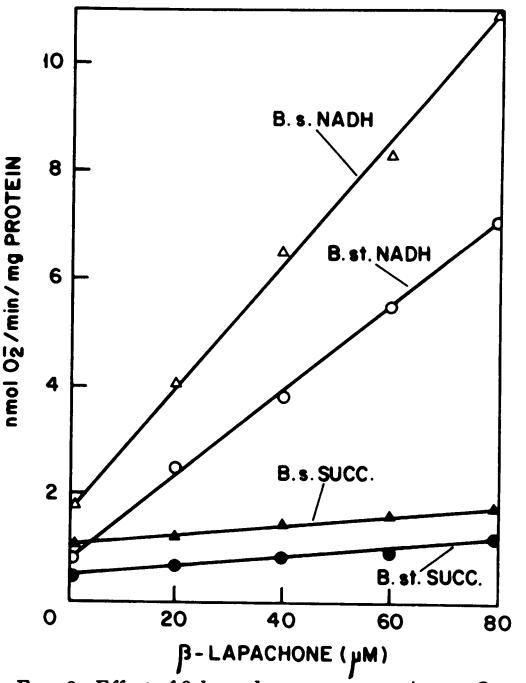

FIG. 2. *Effect of β-lapachone concentration on O_2^- formation by membranes of B. stearothermophilus (B. st.) and B. subtilis (B. s.) in the presence of 40 μM NADH or 50 μM succinate. Experimental conditions as in Fig. 1.*

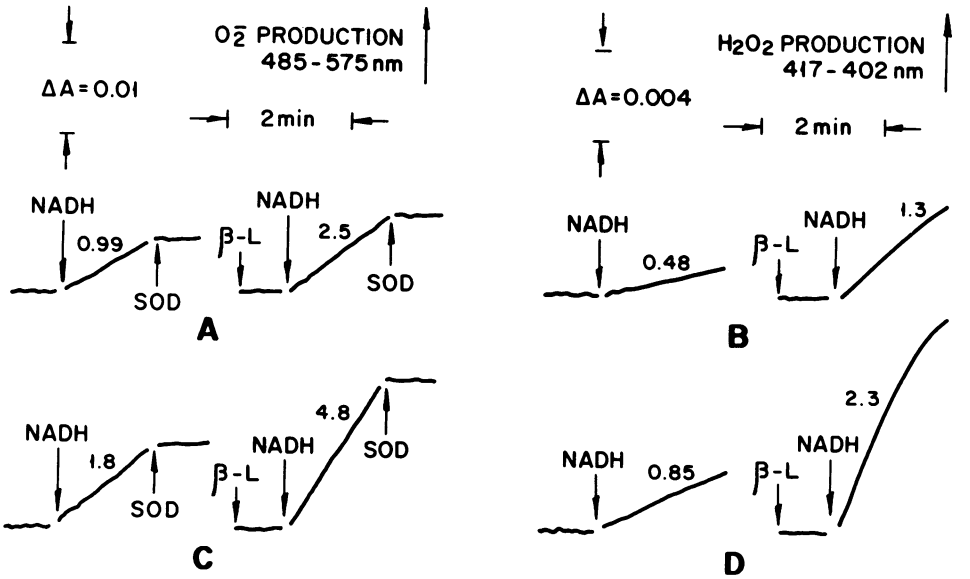

FIG. 1. *Generation of O_2^- and H_2O_2 in membranes from B. stearothermophilus (A and B) and B. subtilis (C and D). The membranes (0.28 mg of protein per ml for B. stearothermophilus and 0.36 mg/ml for B. subtilis) were suspended in a medium containing 0.23 M mannitol, 0.07 M sucrose, 5 mM tris(hydroxymethyl) aminomethane-hydrochloride (pH 7.4), 1 mM ethylenediaminetetraacetate, 2 μM antimycin A, and 1 mM epinephrine (for O_2^- determination) or 0.5 μM horseradish peroxidase (for H_2O_2 determination) in a final volume of 3.0 ml. Concentrations of 40 μM NADH, 20 μM β-lapachone (β-L), and 30 μg of superoxide dismutase (SOD) per ml were added as indicated. Values indicate O_2^- generation or H_2O_2 production in nanomoles per minute per milligram of protein. A, Absorbancy.*

TABLE 2. *Effect of substrates on superoxide anion and hydrogen peroxide formation by bacterial fragments*[a]

Microorganism	Succinate			NADH		
	O^-_2	H_2O_2	O^-_2/H_2O_2	O^-_2	H_2O_2	O^-_2/H_2O_2
B. stearothermophilus	0.45	0.19	2.2	0.97	0.48	2.0
B. subtilis	1.00	0.54	1.9	1.80	0.85	2.1

[a] Experimental conditions as in Fig. 1 and 2.

(7). The inhibition could be reversed or prevented by the addition of cyanide. Heat-inactivated superoxide dismutase did not inhibit adrenochrome formation. An enzymatic reaction was apparently required in this system, since no alteration in the absorbance indicating O_2^- production was observed in the absence of the subcellular fragments from either *B. stearothermophilus* or *B. subtilis*. Moreover, the rate of the reaction was directly proportional to the amount of protein. Similarly, no significant H_2O_2 production was observed in the subcellular fragments upon addition of 20 μM β-lapachone alone (Fig. 1B and D). Further addition of NADH induced an increase in H_2O_2 production 2.5 times greater than that measured in the preparations without β-lapachone.

Figure 2 shows a titration of the effect of β-lapachone on O_2^- production by the bacterial membranes. NADH was more effective than succinate in providing reducing equivalents for O_2^- and H_2O_2 production; a similar specificity has been previously reported in beef heart submitochondrial particles (3). With the *B. subtilis* membranes the rates of O_2^- and H_2O_2 formation were about two times greater than with the membranes from *B. stearothermophilus*. There is a stoichiometric relationship between the O_2^- and H_2O_2 formed in bacterial fragments with either NADH or succinate as the substrate (Table 2).

The possibility that O_2^- and H_2O_2 are important agents in the toxicity of β-lapachone to *B. subtilis* and *B. stearothermophilus* is consistent with the two-times-higher rate of O_2^- and H_2O_2 formation and the two-times-higher sensitivity to β-lapachone in *B. subtilis* as compared with *B. stearothermophilus*. However, this interpretation is not free of criticism, since it assumes that *B. subtilis* and *B. stearothermophilus* do not differ in their contents of superoxide dismutase, catalase, and peroxidase. The enhanced lethality of β-lapachone under aerobic conditions may relate to its generation of O_2^- and H_2O_2 by a cycle of reduction and spontaneous autoxidation.

Formation of O_2^- and H_2O_2 may be considered a likely explanation for β-lapachone toxicity in bacteria. Interaction of O_2^- and H_2O_2 may lead to the formation of a hydroxyl radical and singlet oxygen (13), which in turn starts a free-radical chain reaction that leads to enzyme inactivation and extensive lipid peroxidation (13, 19). Some experimental evidence has been recently provided along these lines by Docampo et al. (5) which showed increased free-radical, O_2^-, H_2O_2, and lipid peroxide formation in *T. cruzi* treated with β-lapachone. Moreover, a correlation between O_2^- production and an inhibitory effect on growth has been found in *T. cruzi* supplemented with a series of 14 β-lapachone derivatives (A. Boveris, A. O. M. Stoppani, R. Docampo, and F. S. Cruz, Comp. Biochem. Physiol., in press).

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