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LIPID PEROXIDATION AND THE GENERATION OF FREE RADICALS AND HYDROGEN PEROXIDE IN β-LAPACHONE TREATED TRYPANOSOMA CRUZI EPIMASTIGOTES

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SUMMARY: Formation of a semiquinone radical as a product of β-lapachone metabolization in Trypanosoma cruzi epimastigotes has been definitely established by electron spin resonance spectroscopy. T. cruzi epimastigotes incubated with β-lapachone showed a marked increase in their activity of H_2O_2 generation. It was also established that T. cruzi epimastigotes undergo lipid peroxidation when incubated with β-lapachone.

INTRODUCTION

β-Lapachone (3,4-dihydro-2,3-dimethyl-2H-naphtho (1,2-b) pyran-5,6-dione) is an antimicrobial agent (1) originally isolated as a contaminant of lapachol preparations (1). In a previous communication (2) we showed that the incubation of Trypanosoma cruzi epimastigotes with β-lapachone caused: a) damage of the nuclear, mitochondrial and cytoplasmic membranes, alterations in the chromatin structure and swelling of the mitochondrion; b) reduction of the respiratory rate and c) inhibition of glucose and pyruvate oxidation. An enhanced H_2O_2 generation in epimastigotes incubated with β-lapachone was also determined by cytochemical and spectrophotometric techniques (3).

In the present work, the formation of free radicals as a result of β-lapachone metabolization in T. cruzi epimastigotes has been definitely established by electron spin resonance (ESR) spectroscopy. In addition we show that T. cruzi epimastigotes undergo lipid peroxidation when incubated with β-lapachone.

MATERIALS AND METHODS

Culture Methods

Trypanosoma cruzi (Y strain) was grown in Warren's liquid medium (4) at 28°C. Six days after inoculation cells were collected by centrifugation and washed with 0.154 M NaCl. The final concentration of epimastigotes was estimated as described before (2).

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Reagents

Horseradish peroxidase (HRP), was obtained from Sigma Chemical Co., St. Louis, Mo.,U.S.A. and β-lapachone was obtained through the Program for the synthesis of Antiparasitic Drugs of Federal University of Rio de Janeiro and was donated by Drs. Antonio Ventura Pinto and Benjamin Gilbert.

ESR Spectroscopy

Epimastigotes were suspended in 0.22 M tris-HCl buffer, pH 7.4 at a concentration of 10^9 cells/ml. Ethanol was used as the β-lapachone solvent. Ethanol itself did not produce measurable free radicals when mixed with the epimastigote suspension. The buffer alone also did not produce free radicals when mixed with β-lapachone. ESR spectra were obtained in a Varian E-9 spectrometer employing the conditions described in the figures. A Varian aqueous sample cell was used. β-lapachone solution as well as the cell suspension mixed with β-lapachone were previously saturated with nitrogen.

Generation of H_2O_2

H_2O_2 generation in epimastigotes was determined by the HRP assay (5) which measures the absorption at 417 nm of the horseradish peroxidase-H_2O_2 Compound II. The reaction mixture contained 2 μM HRP in a buffer consisting of 120 mM KCl and 20 mM potassium phosphate buffer, pH 7.2. An Acta III double beam spectrophotometer (Beckman Instruments, California) was used and all determinations were made at 30°C. Protein was determined by the biuret method (6).

Lipid Peroxidation

The thiobarbituric acid (TBA) reaction was carried out as described by Nakano *et al.* (7). The absorbancy reading was plotted directly rather than being converted to malondialdehyde equivalents.

RESULTS

ESR Spectroscopy

The presence of an ESR signal in a biological sample is construed as evidence for the occurrence of a free radical. If observed, the hyperfine interaction in the spectrum of a complex mixture, can be compared with the signal arising in spectra of the already known free radical. The degree of resolution can provide information on the type and extent of molecular species interaction in the mixture. Free radicals were produced by reduction of β-lapachone both chemically and biologically. Biological reduction of β-lapachone was obtained in suspensions of *T. cruzi* epimastigotes. It was necessary to monitor the signal for a while before maximum intensity developed. The signal grew in intensity in about half an hour and thereafter remained constant (Fig. 1).

The nature of the β-lapachone free radical which arose in the biological system using T. cruzi epimastigotes (Fig. 2) was confirmed by comparison with the spectrum of the chemically reduced β-lapachone (Fig. 2 A). The characteristics of the signal suggests a semiquinone radical. The 5-line hyperfine structure of the β-lapachone semiquinone anion was clearly discernible in both chemically and biologically reduced samples. The spectra were recorded within a few hours after the addition of β-lapachone to cell suspensions. Further observations were made about 20 hours later, the sample being kept at 20°C. After this period there were marked modifications of the spectrum (Fig. 3): a) loss of resolution of the hyperfine structure and b) appearance of broad but well defined lateral wings; the spectrum obtained by chemical reduction does not show modifications, except a decay in intensity.

Generation of H_2O_2

The rate of formation of H_2O_2 in T. cruzi epimastigotes, as detected by horseradish peroxidase Compound II formation (5) is illustrated in Fig. 4. Upon addition of the cells no significant rate of H_2O_2 production was observed. The further addition of β-lapachone caused a marked stimulation of H_2O_2 production.

Lipid Peroxidation

Determination of lipid peroxidation in intact cells was made as described by Nakano et al. (1971). Metabolism of endogenous substrates by epimastigotes resulted in detectable amounts of malondialdehyde (Table 1). Incubation of epimastigotes with β-lapachone increased the amount of malondialdehyde formed. The data presented in Table 1 refer to 3-hr incubation periods but shorter times can be used.

DISCUSSION

The presence of the semiquinone radical as product of β-lapachone metabolization in T. cruzi epimastigotes has been definitely established in the present work by ESR spectroscopy.

Misra and Fridovich (8) have demonstrated that autooxidation of the fully reduced form of certain quinones caused a divalent reduction of oxygen generating H_2O_2, whereas with semiquinones there occurred the univalent reduction of oxygen generating O_2^-. McCord and Fridovich (9) have described a superoxide dismutase, which scavenges the superoxide radical and produces H_2O_2:

$$O_2^- + O_2^- + 2\,H^+ \longrightarrow H_2O_2 + O_2 \quad (1)$$

As O_2^- is an unstable free radical which appears only in very minor amounts at a time (10), the superoxide anion generated by the semiquinone might disproportionate to H_2O_2 and O_2. This is in accordance with the observed production of H_2O_2 in epimastigotes (Fig. 4 and (3)). In 1934 Haber and Weiss (11) proposed the following mechanism for the disproportionation of hydrogen peroxide:

$$H_2O_2 + O_2^- \longrightarrow OH^- + OH^\bullet + O_2 \quad (2)$$

Kellog and Fridovich (12) recently suggested that the Haber-Weiss reaction is a source of singlet oxygen (O_2^*). On thermodynamic grounds, Koppenol (13) showed it to be possible and postulated that this form of oxygen can react with various groups, for instance unsaturated bonds, having a destructive effect on proteins.

It has been suggested that peroxidation of subcellular membranes is initiated by a hydroxyl radical (14). The superoxide anion, generated by β-lapachone semiquinone oxidation, would then dismutate to form hydrogen peroxide (reaction (1)), which in turn would react with additional superoxide to form the hydroxyl radical (reaction (2)). Hydroperoxide formation can be initiated as the hydroxyl radical removes a hydrogen atom from the α-methylene carbons of the unsaturated fatty acids. In addition, singlet oxygen, generated by the non-enzymatic dismutation of superoxide (reaction (2)) will readily react with the unsaturated fatty acids to form hydroperoxides allowing the subsequent oxidative reactions that results in malondialdehyde production.

The modifications of the ESR spectrum of the biological system awaits further interpretation, but they can probably be assigned either to radicals

attached to the cell membrane or to some other radical arising in the system, such as that produced after the direct reaction of oxygen and lipids (15). Other processes cannot be excluded. A characteristic three-line electron spin resonance signal has been reported to be observed in normal tissues that had decayed on standing for 1 or 2 days at room temperature and it was attributed to the denatured form of NO-hemoproteins (16).

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TABLE I. Effect of β-lapachone on lipid peroxidation in Trypanosoma cruzi epimastigotes.

Incubation for 3 hr was carried out with T. cruzi epimastigotes (11 mg dry weight) at 37°C and pH 7.4 in 0.08 M sodium phosphate buffer containing the concentration of β-lapachone mentioned in the table, in a total volume of 5 ml.

Added substance (µg/ml)	A 532 nm
None	0.060
β-Lapachone 5	0.120
10	0.220
25	0.600

LEGENDS TO FIGURES

Fig. 1. Time course of β-lapachone free radical arising after addition of the suspension of T. cruzi epimastigotes. X-Band (3 cm) ESR spectra were recorded at 20 gauss/min. The modulation amplitude was 6.3 gauss at a frequency of 100 kHz. Each spectrum was recorded 135 sec after the previous one, the first being recorded 5 min after the mixture.

Fig. 2. X-Band (3 cm) ESR spectra of β-lapachone free radical (A) Chemically reduced: β-lapachone at a concentration of 2 mg/ml in tris medium was deaereated with nitrogen and reduced by addition of a solution of sodium borohydride to a concentration of 40 µg/ml. The spectrum was recorded at 40 gauss/min. The modulation amplitude was 0.63 gauss at a frequency of 100 KHz. (B) Biologically reduced: to a suspension of T. cruzi in tris medium β-lapachone was added to a final concentration of 2 mg/ml and was deaereated with nitrogen. The spectrum was recorded at 0.1 gauss, 100 KHz, with high gain, high time constant and slow sweep.

Fig. 3. X-Band (3 cm) ESR spectra of β-lapachone free radical (A) recorded 20 hr after the mixture of epimastigotes suspension and β-lapachone. The spectrum was recorded at 5 gauss/min, the modulation amplitude was 1 gauss at a frequency of 100 KHz. In relation to the spectrum shown in Fig. 2 there was a loss of the 5-line hyperfine structure and appearance of broad but well defined lateral wings. (B) The same spectrum recorded at 25 gauss/min, 100 kHz with lower modulation to show a better resolution of the lateral wings.

Fig. 4. H_2O_2 generation in intact T. cruzi epimastigotes as measured by HRP Compound II formation. T. cruzi epimastigotes (0.2 mg/ml protein) were suspended in a medium containing 0.120 M KCl, 20 mM potassium phosphate buffer at pH 7.2 and 30°C.

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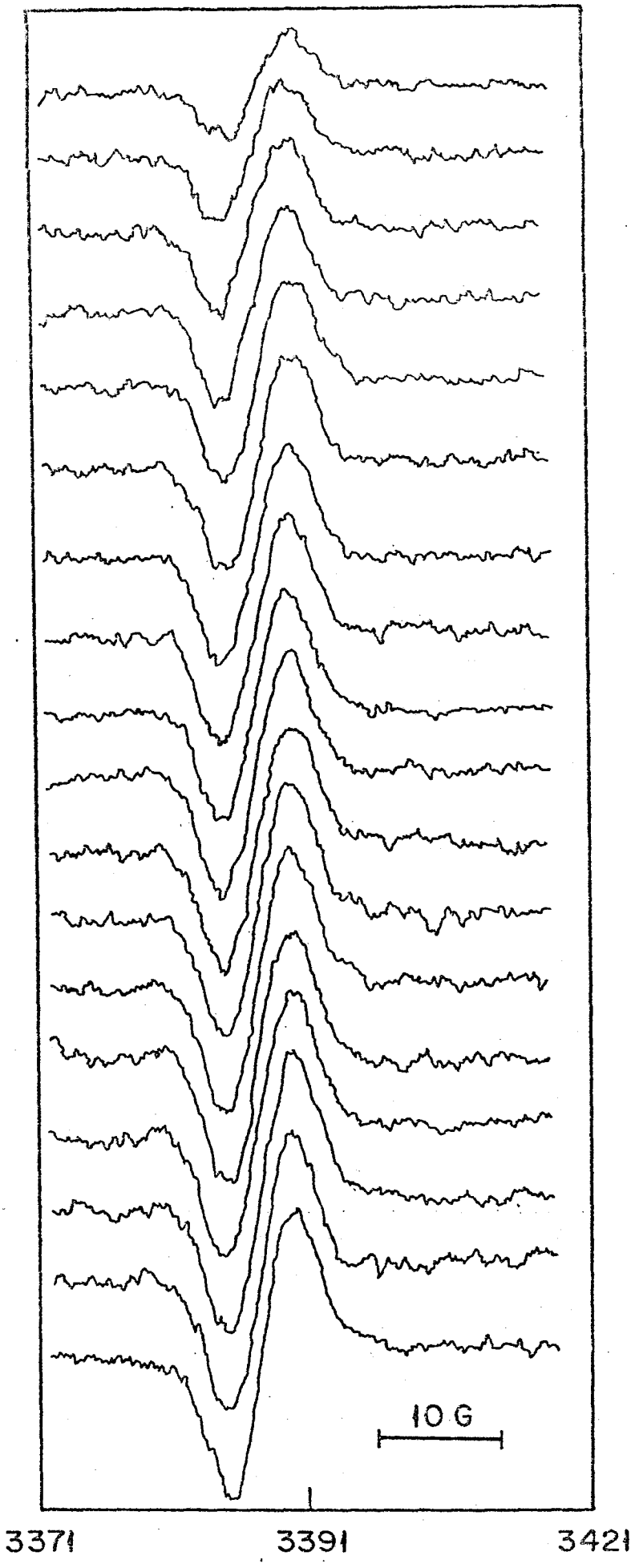


Fig. 1

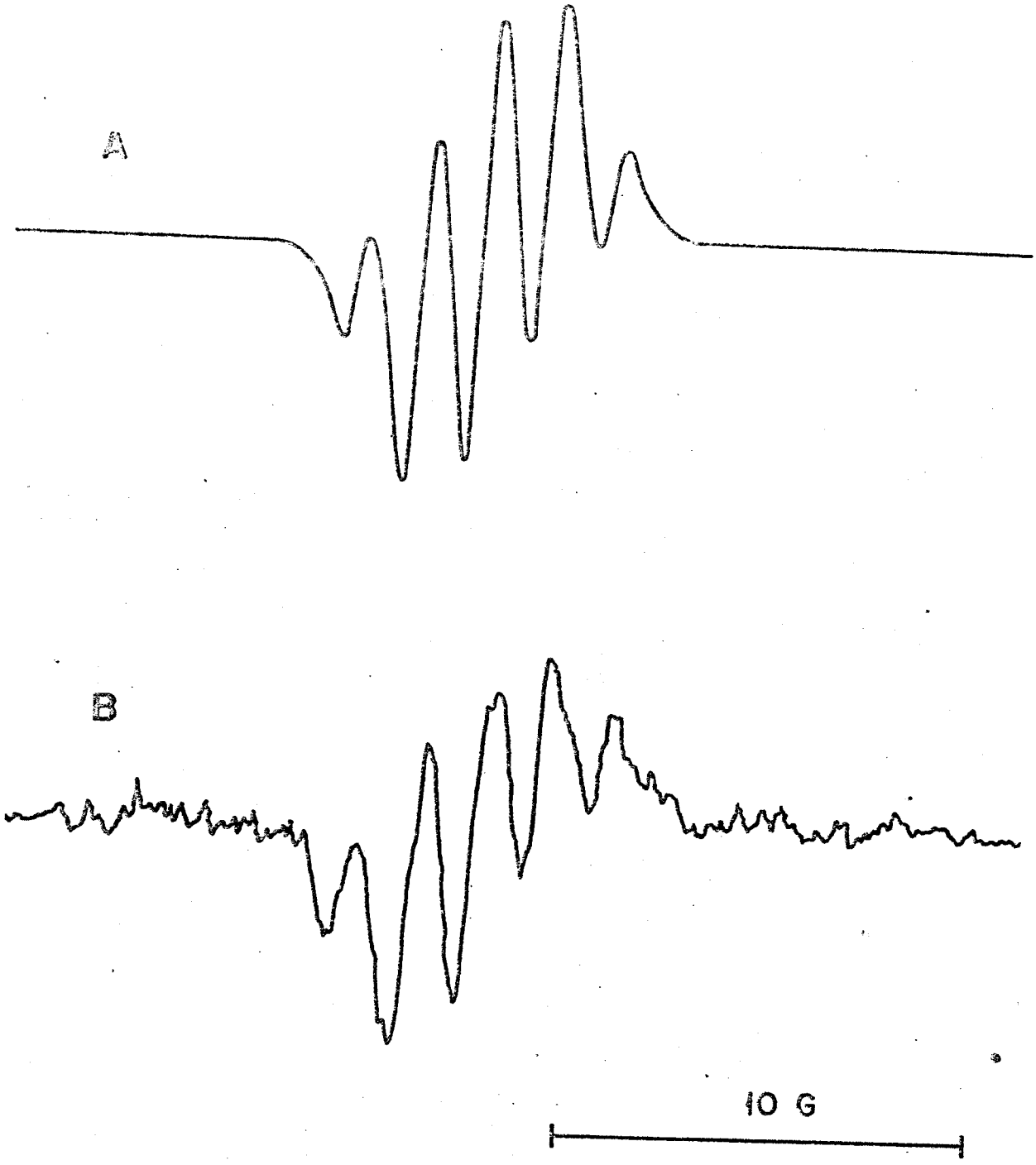


Fig. 2

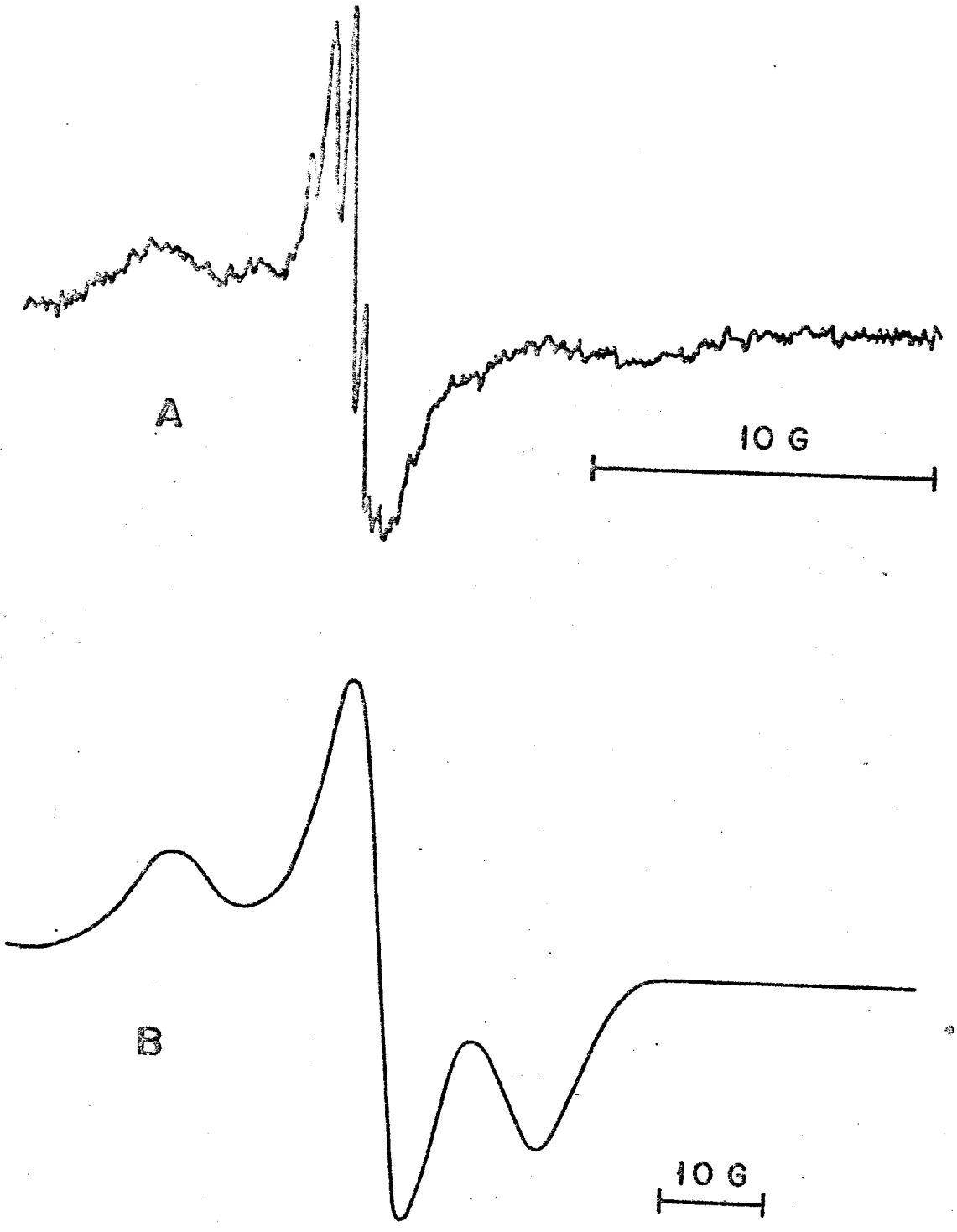
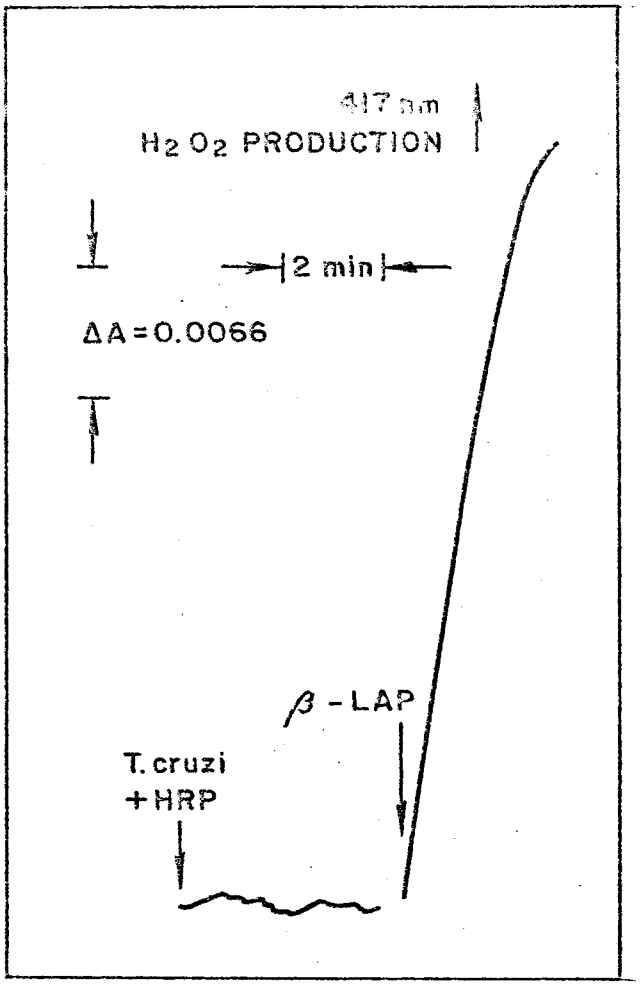


Fig. 3



.Fig. 4