Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 35 (1978)
Heft: 3

Artikel: Generation of free radicals from phenazine methosulfate in "Trypanosoma cruzi" epimastigotes
Autor: Docampo, R. / Cruz, F.S. / Muniz, R.P.A.
DOI: https://doi.org/10.5169/seals-312384

Acta Tropica 35, 221–228 (1978)

Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Brasil, and
Centro Brasileiro de Pesquisas Físicas, Rio de Janeiro, Brasil

Generation of free radicals from phenazine methosulfate in *Trypanosoma cruzi* epimastigotes

R. Docampo[1], F. S. Cruz, R. P. A. Muniz, D. M. S. Esquivel,
M. E. L. de Vasconcellos

Summary

A significant growth inhibition of *Trypanosoma cruzi* epimastigotes by phenazine methosulfate (PMS) was observed in Warren's medium. This toxic activity could be related to the following parameters: a) formation of phenazinium free radical, b) generation of superoxide anion in intact cells incubated with PMS, and c) PMS also increased significantly the rate of O_2^- generation in epimastigotes mitochondrial and microsomal fractions using NADH as electron donor.

Key words: Trypanosoma cruzi; epimastigotes; phenazine methosulfate; growth inhibition.

Introduction

Phenazine and related compounds, including *N*-alkylated derivatives are oxidized or reduced with the formation of semiquinoid intermediates (Zaugg, 1964). 5-Methylphenazinium methylsulfate (usually referred to as phenazine methosulfate [PMS]) was introduced as an electron acceptor in mitochondria by Kearney and Singer (1956). Electron spin resonance (ESR) studies of succinic dehydrogenase in which PMS acted as an electron acceptor showed the formation of free radicals (Hollocher and Commoner, 1961; King et al., 1961) suggesting that in this system the dye may be partially reduced (King, 1963). When PMS is added to live suspension of *Escherichia coli* or *Bacillus megaterium*, the

[1] Permanent address: Instituto de Química Biologica, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

Correspondence: Dr. Fernando S. Cruz, Instituto de Microbiologia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Cidade Universitária, Bloco 1, Rio de Janeiro, 20000 – Brasil

corresponding free radical is produced intracellularly and the viability of the cells is affected (White and Dearman, 1965). In previous studies from our laboratory we have postulated that a free radical arising from a 1,2-naphthoquinone (β-lapachone) induced the production of superoxide anion and hydrogen peroxide which could explain the toxicity of the drug (Docampo et al., 1977; Cruz et al., 1978; Docampo et al., 1978). Other drugs have also been investigated, and in the present communication we report the biological generation of free radicals from PMS by *T. cruzi* epimastigotes.

Materials and methods

Culture methods. T. cruzi (Y strain) was grown on Warren's liquid medium (Warren, 1960) at 28° C. Six days after inoculation the cells were collected by centrifugation and washed with 0.15 M NaCl solution. Growth of organisms was estimated using a Neubauer counting chamber. Fresh media were inoculated with 0.5 to 1.0×10^7 cells/ml. The drug was added aseptically as ethanolic solution. The volume of ethanolic drug solution which was added to parasite cultures was always less than 20 μl/ml of culture medium. This concentration of ethanol did not affect the growth of epimastigotes.

Chemicals. Analytical grade reagents were used throughout the experiments. L-epinephrine, erythrocyte superoxide dismutase (SOD) and phenazine methosulfate (PMS) were obtained from Sigma Chemical Co., St. Louis, Mo., USA.

Mitochondrial and microsomal preparations. T. cruzi epimastigotes were disrupted by freezing (at –70° C) and thawing (at 2–4° C) three times. The suspension was homogenized by passing it three times through a hypodermic needle (gauge 24). The homogenates were suspended in 0.23 M manitol, 0.07 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.2 at 8 mg protein/ml and fractionated in the Sorvall RC-2B centrifuge at 4° C. The fractions obtained were: a) the nuclear-flagellar fraction (sedimented at $480 \times g$ for 10 min; the fluffy layer was reincorporated to the supernatant); b) the mitochondrial fraction (sedimented at $12{,}000 \times g$ for 10 min); c) the microsomal fraction (sedimented at $105{,}000 \times g$ for 60 min); d) the supernatant.

Electron spin resonance spectroscopy. T. cruzi epimastigotes were suspended in 0.22 M Tris-HCl buffer, (pH 7.4) at a concentration of 10^9 cells/ml. Solutions of PMS were made in ethanol. Ethanol itself did not produce measurable free radicals when mixed with the epimastigote suspension. Also, the Tris-HCl buffer alone did not produce free radicals when mixed with PMS. ESR spectra were obtained in a Varian E-9 spectrometer using the conditions described in the Figures. A Varian aqueous sample cell was used. PMS solution as well as the cell suspension mixed with PMS were previously saturated with nitrogen.

Determination of superoxide generation. O_2^- production was determined by the adrenochrome assay (Misra and Fridovich, 1972) measuring the absorption at 485–575 nm and using an absorption coefficient (ε) of 2.96 $nM^{-1}\ c\ m^{-1}$ (Cadenas et al., 1977). The reaction mixture contained 1 mM epinephrine in the saline solution described before (Docampo et al., 1977). An Aminco-Chance double beam spectrophotometer (American Instrument Company, Silver Springs, Maryland, USA) was utilized. All determinations were made at 30° C. Protein was determined by the biuret method (Gornall et al., 1949).

Results

ESR spectroscopy. When an ethanolic solution of PMS was added to a suspension of *T. cruzi* epimastigotes and the cells were placed in an aqueous sample cell for obervation in an ESR spectrometer, the signal of the semiqui-

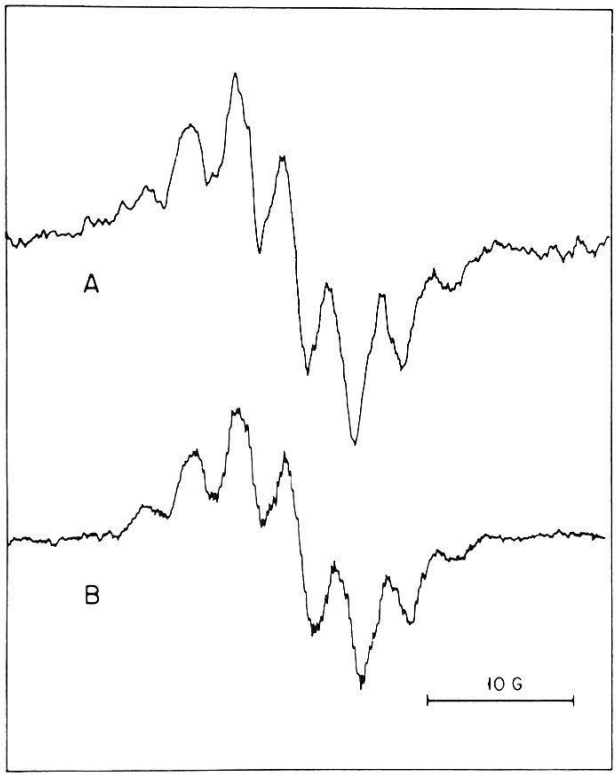

Fig. 1. X-Band (3 cm) ESR spectra of phenazine methosulfate free radical: (A) Chemically reduced: PMS at a concentration of 2 mg/ml in 0.22 M Tris-HCl buffer pH 7.4 was deaerated with nitrogen and reduced by addition of sodium borohydride to a concentration of 40 μg/ml. The spectrum was recorded at 6.25 gauss/min. The modulation amplitude was 0.50 gauss at a frequency of 100 KHz. (B) Biologically reduced: to a suspension of *T. cruzi* in Tris medium, PMS was added to a final concentration of 2 mg/ml and was deaerated with nitrogen. The spectrum was recorded at 0.50 gauss at a frequency of 100 KHz.

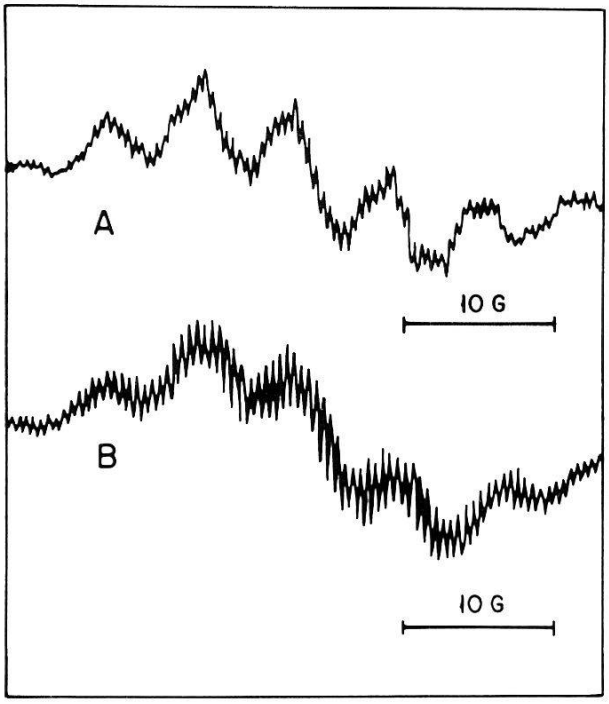

Fig. 2. X-Band (3 cm) ESR spectra of phenazine methosulfate free radical: (A) Recorded 50 min after the mixture of the *T. cruzi* epimastigote suspension and PMS. The spectrum was recorded at 0.66 gauss/min, the modulation amplitude was 0.16 gauss at a frequency of 100 KHz. (B) Same spectrum recorded at 2.50 gauss/min showing a better resolution of the signal.

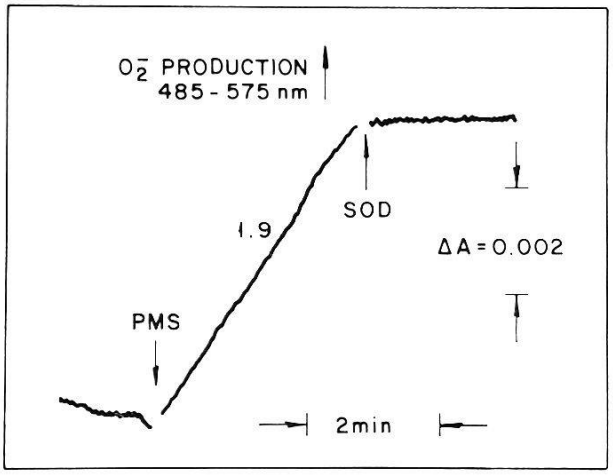

Fig. 3. Effect of phenazine methosulfate on O_2^- production in *T. cruzi* epimastigotes. Cells (0.3 mg protein/ml) were suspended in the saline solution described in Table 1. 10 μg/ml PMS and 0.1 mg superoxide dismutase were added as indicated.

Table 1. Superoxide anion diffusion in *T. cruzi* epimastigote suspension in presence of phenazine methosulfate*

Phenazine methosulfate (μg/ml)	O_2^- diffusion (nmol/min/mg protein)	
	without SOD	with SOD (0.1 mg)
None	0.00	0.00
3.3	0.48	0.03
5.0	0.72	0.05
6.6	1.08	0.04
10.0	1.90	0.01

* Incubation was performed as described in the text. The reaction medium consisted of 35 mM Tris-HCl buffer (pH 7.2), 5.0 mM phosphate buffer (KH_2PO_4-Na_2HPO_4, pH 7.2) 0.05 M NaCl, and 0.1 M KCl, 1 mM epinephrine, and cell suspension equivalent to 1 mg protein in a final volume of 3 ml.

noid derivative soon developed (Fig. 1 B). Since no substrate was present, ethanol acted as a source of electrons. The 7-line hyperfine structure of the phenazinium radical was clearly discernible and was similar to the signal produced by the chemical reduction of PMS (Fig. 1 A). The signal grew in intensity for about 50 min and thereafter remained nearly constant (Fig. 2) for more than 24 h in both chemically and biologically reduced samples.

Generation of superoxide anion. The rate of formation of O_2^- in *T. cruzi* epimastigotes as detected by the adrenochrome formation is illustrated in Fig. 3. Upon addition of cells no significant rate of O_2^- production was observed. Further addition of different concentrations of PMS caused a marked stimulation of O_2^- production (Fig. 3 and Table 1) specifically inhibited by superoxide dismutase. This inhibition could be reversed or prevented by the addition of cyanide, an inhibitor of SOD (Fridovich, 1974). Heat inactivated

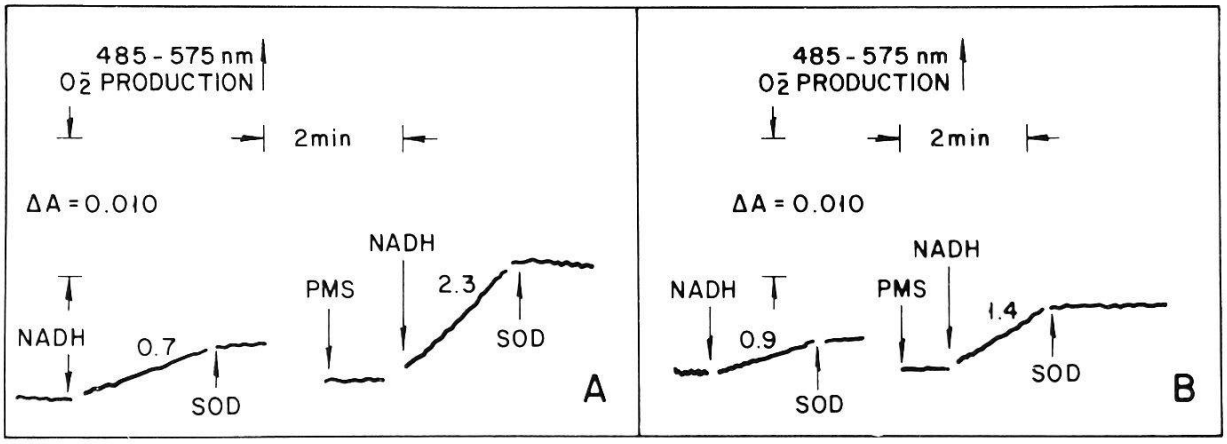

Fig. 4. O_2^- generation in mitochondrial (A) and microsomal (B) fractions from *T. cruzi* epimastigotes. The fractions (mitochondria 0.3 mg protein/ml; microsomes: 0.5 mg protein/ml) were suspended in 0.23 M manitol, 0.07 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.2 (mitochondria) or 0.13 KCl and 20 mM potassium phosphate, pH 7.2 (microsomes) and 1 mM epinephrine. 40 μM NADH, 5 μg/ml phenazine methosulfate and 0.1 mg superoxide dismutase were added as indicated. Values indicate O_2^- generation in nmol/min/mg protein.

SOD did not inhibit adrenochrome formation. An enzymatic reaction was apparently required in this system since no alteration in the absorbance was observed in the absence of cells, the rate of the reaction being directly proportional to the amount of cell protein.

The rate of formation of O_2^- in the mitochondrial fraction of *T. cruzi* epimastigotes is illustrated in Fig. 4 A. Upon addition of PMS alone no significant rate of O_2^- production was observed. Further addition of NADH caused a greater stimulation of O_2^- production than in the preparations without PMS. This O_2^- production was specifically inhibited by SOD. This inhibition could be reversed or prevented by the addition of cyanide. Heat inactivated SOD did not inhibit adrenochrome formation. No alteration in the absorbance was observed in the absence of the fraction and the rate of the reaction was directly proportional to the amount of cell protein. Fig. 5 A shows the effect of PMS concentration on O_2^- formation by a *T. cruzi* mitochondrial fraction.

The rate of formation of O_2^- in the microsomal fraction of the epimastigotes is illustrated in Fig. 4 B. Upon addition of PMS alone no significant rate of O_2^- production was observed. Further addition of NADH caused a greater stimulation of O_2^- production than in the preparations without PMS. This O_2^- production was specifically inhibited by SOD. This inhibition could be reversed or prevented by the addition of cyanide. Heat inactivated SOD did not inhibit adrenochrome formation. No alteration in the absorbance was observed in the absence of the fraction and the rate of the reaction was directly proportional to the amount of cell protein. Fig. 5 B shows the effect of PMS concentration on O_2^- formation by the *T. cruzi* microsomal fraction.

Inhibition of growth. The growth curve of *T. cruzi* in Warren's liquid medium and the effect of different concentrations of PMS is shown in Fig. 6.

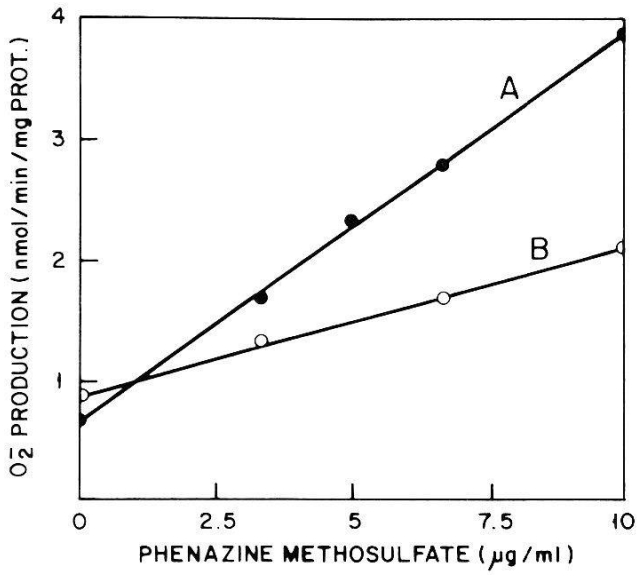

Fig. 5. Effect of phenazine methosulfate concentration on O_2^- formation by the mitochondrial (A) and microsomal (B) fractions of *T. cruzi*. Experimental conditions as in Fig. A.

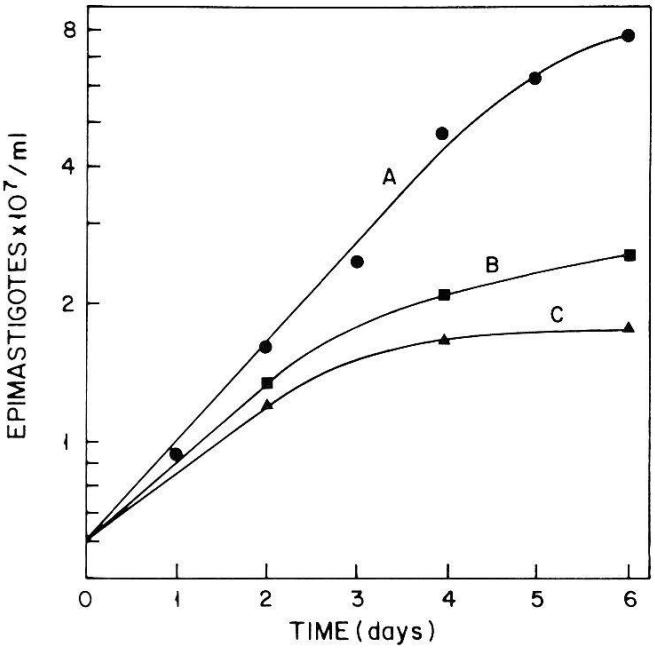

Fig. 6. Effect of phenazine methosulfate on the growth of *T. cruzi* epimastigotes in Warren's liquid medium (6). Growth of organisms was estimated using a Neubauer counting chamber. Four determinations for each concentration were made. A: no additions; B: 2.5 μg/ml PMS; C: 15 μg/ml PMS.

A 60% inhibition with 2.5 μg/ml of PMS could be observed. At this concentration the parasite number doubled two times during the culture period. At 15 μg/ml a higher growth inhibition was obtained. Attempts to subculture *T. cruzi* epimastigotes growth at these concentrations of PMS in fresh media were not successful. Only cell detritus was observed under the light microscope after 3–4 days of inoculation.

Discussion

It was proposed (White and Dearman, 1965) that the bactericidal activity of quinones is due to the formation of free radicals. However, it was postulated (Ishizu et al., 1968) that further reaction of the semiquinone is necessary for the lethal activity and that only a few semiquinones are capable of this reaction. We have suggested in the case of β-lapachone-treated epimastigotes of *T. cruzi* that a reaction may occur between the semiquinone and oxygen with the formation of O_2^- and H_2O_2 (Docampo et al., 1978). It is known (Ishizu et al., 1968) that PMS is rapidly and non-enzymatically reduced by NADH or NADPH. Therefore the occurrence of a phenazinium free radical does not imply the participation of enzymes. In addition, the persistence of PMS radicals when the reduction is carried out biologically may be due to a direct or indirect inactivation of cellular metabolism by the drug, thus rendering the cells unable to transferring additional electrons to PMS, as observed in other microorganisms (White and Dearman, 1965).

A rapid O_2^- formation was obtained in intact cells as well as in the mitochondrial and microsomal fraction of *T. cruzi* epimastigotes in presence of PMS. NADH was necessary as electron donor for O_2^- formation in the mitochondrial and microsomal fractions, and this may be construed as an explanation for PMS toxicity against these cells since such intermediate is highly toxic to biological systems. O_2^- is converted to H_2O_2 either spontaneously or by SOD. O_2^- and H_2O_2 react nonenzymatically to form the hydroxyl radical (Haber and Weiss, 1934) and singlet oxygen (Koppenol, 1976), and these products of the partial reduction of oxygen can initiate free-radical reactions which lead to the peroxidation of polyunsaturated fatty acids of membrane lipids (Fong et al., 1973; Tappel, 1973).

A substantial amount of intracellular level of hydrogen peroxide caused by a lack of catalase and the homolytic scission of this oxygen-reactive intermediate to hydroxyl radicals, have been proposed as factors in the toxicity of heme compounds in *Trypanosoma brucei* bloodstream forms (Meshnick et al., 1977). Since *T. cruzi* epimastigotes contain peroxidase but not catalase (Docampo et al., 1976) the intracellular level of H_2O_2 presumably depends on both: a) the rate of H_2O_2 generation and b) the supply of hydrogen donors for the peroxidase reaction. Imbalance in this process might accompany administration of drugs such as PMS. The present results suggest the potential use of free radicals generating drugs for the chemotherapy of Chagas' disease.

Acknowledgments. We thank Dr. L. R. Travassos for help with the manuscript. This work was supported by the World Health Organization (WHO), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Financiadora de Estudos e Projetos (FINEP, Convênio No. 362), Brasil.

1 Cadenas E., Boveris A., Ian Ragan C., Stoppani A. O. M.: Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome *c* from beef heart mitochondria. Arch. Biochem. Biophys. *180*, 248–257 (1977).
2 Cruz F. S., Docampo R., de Souza W.: Effect of β-lapachone on hydrogen peroxide production in *Trypanosoma cruzi*. Acta trop. (Basel) *35*, 35–40 (1978).
3 Docampo R., Boiso J. F., Boveris A., Stoppani A. O. M.: Localization of peroxidase activity in *Trypanosoma cruzi* microbodies. Experientia (Basel) *32*, 972–975 (1976).
4 Docampo R., Lopes J. N., Cruz F. S., de Souza W.: *Trypanosoma cruzi:* Ultrastructural and metabolic alterations of epimastigotes by β-lapachone. Exp. Parasit. *42*, 142–149 (1977).
5 Docampo R., Cruz F. S., Boveris A., Muniz R. P. A., Esquivel D. M. S.: Lipid peroxidation and the generation of free radicals, superoxide anion and hydrogen peroxide in β-lapachone-treated *Trypanosoma cruzi* epimastigotes. Arch. Biochem. Biophys. *186*, 36–41 (1978).
6 Fong K. L., McCay P. B., Poyer J. L., Keele B. B., Misra H.: Evidence that peroxidation of lysosomal membrane is initiated by hydroxyl free radicals produced during flavin enzyme activity. J. biol. Chem. *248*, 7792–7797 (1973).
7 Fridovich I.: Superoxide dismutase. Advanc. Enzymol. *41*, 35–97 (1974).
8 Gornall A. G., Bardawill C. S., David M. N.: Determination of serum proteins by means of the biuret reaction. J. biol. Chem. *177*, 751–766 (1949).
9 Haber F., Weiss J.: The catalytic decomposition of hydrogen peroxide by iron salts. Proc. roy. Soc. London A *147*, 332–351 (1934).
10 Hollocher T. C. jr., Commoner B.: An electron spin resonance analysis of the mechanism of succinic dehydrogenase activity. Proc. nat. Acad. Sci. (Wash). *47*, 1355–1374 (1961).
11 Ishizu K., Dearman H. H., Huang M. T., White J. R.: Electron paramagnetic resonance observations on biogenic semiquinone and 5-methylphenazinium radicals. Biochim. biophys. Acta (Amst.) *165*, 283–285 (1968).
12 Kearney E. B., Singer T. P.: Studies on succinic dehydrogenase. I. Preparation and assay of the soluble dehydrogenase. J. biol. Chem. *219*, 963–975 (1956).
13 King T. E.: Reconstitution of respiratory chain enzymes systems. XI. Use of artificial electron acceptors in the assay of succinate dehydrogenating enzymes. J. biol. Chem. *238*, 4032–4036 (1963).
14 King T. E., Howard R. L., Mason S. H.: An electron spin resonance study of soluble succinic dehydrogenase. Biochem. biophys. Res. Commun. *5*, 329–333 (1961).
15 Koppenol W. H.: Reactions involving singlet oxygen and the superoxide anion. Nature (Lond.) *262*, 420–421 (1976).
16 Meshnick S. R., Kwang-Poo Chang, Cerami A.: Heme lysis of the bloodstream forms of *Trypanosoma brucei*. Biochem. Pharmacol. *26*, 1923–1928 (1977).
17 Misra H. P., Fridovich I.: The univalent reduction of oxygen by reduced flavins and quinones. J. biol. Chem. *247*, 188–192 (1972).
18 Tappel A. L.: Lipid peroxidation damage to cell components. Fed. Proc. *32*, 1870–1874 (1973).
19 Warren L.: Metabolism of *Schizotrypanum cruzi* Chagas. I. Effect of culture age and substrate concentration on respiratory rate. J. Parasit. *46*, 529–539 (1960).
20 White J. R., Dearman H. H.: Generation of free radicals from phenazine methosulfate, streptonigrin and rubiflavin in bacterial suspensions. Proc. nat. Acad. Sci. (Wash.) *54*, 887–891 (1965).
21 Zaugg W. S.: Spectroscopic characteristics and some properties of *N*-methylphenazinium methyl sulfate (phenazine methosulfate) and pyocyanine at the semiquinoid level. J. biol. Chem. *239*, 3964–3970 (1964).