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The photodynamic action of rose bengal on *Trypanosoma cruzi*

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Summary

In the presence of light and oxygen, rose bengal causes oxidative damage to *Trypanosoma cruzi*. The production of lipid hydroperoxides was demonstrated by thin-layer chromatography, and severe ultrastructural alterations compatible with an increased permeability of the cells, which led to gradual osmotic swelling and ultimately to lysis, were observed by electron microscopy. As a result of this treatment, the infectivity of *T. cruzi* trypomastigotes in mice was abolished. In addition, under anaerobic conditions, rose bengal was found to undergo a one-electron reduction in intact *T. cruzi* epimastigotes to produce a carbon-centered free radical as demonstrated by electron spin resonance spectroscopy. The formation of this radical was also enhanced by light.

Key words: *Trypanosoma cruzi;* rose bengal; singlet oxygen; free radicals; lipid peroxidation.

Introduction

Intracellular reduction followed by autoxidation yielding superoxide anion ($\dot{O}_2^-$) and hydrogen peroxide (H_2O_2) has been suggested as the mode of action of several trypanocidal agents (reviewed in Docampo and Moreno, 1984). Thus, naphthoquinones (Docampo et al., 1977, 1978a, 1978b; Cruz et al., 1978), phenazine methosulfate (Docampo et al., 1978c), and nitroheterocyclic drugs (Docampo and Stoppani, 1979; Docampo et al., 1981) have been shown to act in intact *Trypanosoma cruzi* or extracts as electron carriers between NADH or NADPH and oxygen with concomitant production of either $\dot{O}_2^-$ or H_2O_2. Moreover, oxygen reduction products have been implicated in the mechanism of cytotoxicity of phagocytic cells against *T. cruzi* (Nathan et al.,

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1979; Docampo et al., 1982, 1983a). Although evidence is accumulating on the cytotoxicity of these oxygen reduction products to *T. cruzi*, little is known about the destructive potential of the oxygen excitation product, singlet oxygen ($^1O_2$). Singlet oxygen has been suggested to be involved in many biological processes. Since $^1O_2$ can be formed in the interaction of $\dot{O}_2^-$ and H_2O_2 (Kellog and Fridovich, 1975, 1977; Koppenol, 1976; Khan, 1976; Krinsky, 1977), it has been proposed to be formed during such processes as the antimicrobial activity of phagocytic cells (Rosen and Klebanoff, 1977), inflammation (Fantone and Ward, 1982), redox cycling of quinone drugs, cytostatic agents, and nitroheterocyclic compounds (Kappus and Sies, 1981), and enzymatic reactions such as those catalyzed by xanthine oxidase (Kellog and Fridovich, 1975) and lactoperoxidase (Kanovsky, 1983).

Since dyes absorbing light are among the few natural systems that permit us to study the effects of $^1O_2$ production, we decided to study the effects of rose bengal, a known singlet oxygen producer (Houba-Herin et al., 1982), on *T. cruzi* structure and viability.

Materials and Methods

Culture methods. Trypanosoma cruzi culture forms (Y strain) were grown at 28° C in the liquid medium described by Warren (1960). Five days after inoculation, cells were collected by centrifugation and washed twice with 0.154 M NaCl. The final concentration of epimastigotes was estimated as described before (Docampo et al., 1977).

Isolation of bloodstream forms (Docampo et al., 1981) from mice was made as described in the reference. The final concentration of the cells was estimated by counting in a Neubauer chamber.

Chemicals. Rose bengal was obtained from Eastman Kodak. Other reagents were of analytical grade.

Microscopy. T. cruzi cells were suspended in a saline solution containing 0.154 M NaCl and 0.15 M phosphate buffer (KH_2PO_4–Na_2HPO_4), pH 7.4 (PBS). The final concentration was adjusted to 1×10^8 cells/ml. The cells were preincubated in the dark at different concentrations of rose bengal. Preincubation was done for 60 min in a water bath at 30° C with constant shaking. After preincubation, the cells were centrifuged at 5000 *g* for 10 min and resuspended to a final concentration of 1×10^8 cells/ml in PBS. The absorbance at 541 nm of the supernatants obtained after 0 and 60 min of preincubation was measured against appropriate blanks in a Beckman Acta III spectrophotometer to determine the incorporation of the dye into the cells. The resuspended cells were then illuminated for 10 min with a GE PAR 38 incandescent lamp (cool beam type) of 150 W at a distance of 25 cm. The fluence rate at this distance was 1400 μW/cm^2, and the wavelength was over 400 nm. To avoid an increase in the temperature, a filter of water (2 cm) was intercalated between the light source and the samples. Light microscopy photographs were obtained using Zeiss-Nomarski differential interference contrast microscopy. After incubation, the cells were centrifuged at 5000 *g* for 10 min, washed twice in PBS and then fixed in 2.5% glutaraldehyde-PBS for 60 min at room temperature. After a thorough rinse in PBS, cells were postfixed in 1% OsO_4–PBS at 4° C, dehydrated in ethanol, and embedded in Epon. Ultra thin sections were made in an LKB Ultratome III ultramicrotome. Sections were stained with uranyl acetate and lead citrate and observed in an AEI EM 6-B electron microscope with a 50 μm objective aperture operating at 60 kV.

Lipid analysis. Samples of cell suspensions containing 5×10^9 cells (equivalent to 1.5 g wet weight) were centrifuged at 5000× *g* for 10 min and extracted with chloroform/methanol (2:1) (30 ml/g of cells, wet weight), by the method of Folch et al. (1957). Aliquots of these extracts were

treated as described by Stahl (1958) and chromatographed on Silica Gel, type G, thin layer sheets as described (Stahl, 1958). Peroxides were visualized by the starch-iodide procedure of Stahl (1958).

Electron spin resonance spectroscopy. Observations were made at room temperature (about 24° C) with a Varian E-9 spectrometer using an E-238 TM_{110} cavity equipped with a window to illuminate the sample under the conditions described in Results. The 3-ml incubations contained 1 mM rose bengal and the epimastigotes (25 mg protein/ml) in PBS. The protein concentration was determined as described previously (Gornall et al., 1949). An ELMO-CV-II slide projector with a tungsten lamp of 150 W and light with wavelengths greater than 400 nm was used. The slide projector was at a distance of 30 cm from the front wall of the microwave cavity. The fluence rate at this distance was 1300 $\mu W/cm^2$.

Assay of infectivity in mice. T. cruzi trypomastigotes incubated as described before were injected intraperitoneally into mice (4×10^6 trypomastigotes in 0.2 ml of PBS to each mouse). Ten mice were used for each dye concentration and ten for the controls. The course of parasitemia was determined in the 5th and 7th day and every 5 subsequent days by counting the number of parasites present in one drop of fresh tail blood. Mortality rates were also recorded.

Results

After 60 min of preincubation in the presence of different concentrations of dye, a linear relationship of intracellular accumulation to the external concentration of rose bengal was found without any indication of saturation kinetics over a wide concentration range (5–500 μM). This may suggest the entry of rose bengal by diffusion, and not by active transport, in trypanosomes. No alterations were observed in preincubations in the dark containing low concentrations of dye (5–100 μM). However, an increased loss of motility without any apparent morphological alteration was observed in cells preincubated in the presence of higher concentrations of dye (200–500 μM). When cells preincubated with 10 or 20 μM rose bengal were exposed to room light or the light of the optical microscope (fluence rate at 10 cm of 600 $\mu W/cm^2$), a number of cells turned rounded after 2–3 min (Fig. 1b), although they were still mobile. After 10 min of illumination of these cells, all the cells became rounded and immobile (Fig. 1c). No significant alterations were observed by light microscopy upon illumination of the cells preincubated at a lower concentration of rose bengal (5 μM). Ultrastructural studies revealed cellular damage to the epimastigotes after incubation with rose bengal. Control cells incubated in medium alone remained intact and mobile throughout the experiment (Figs. 1a and 2a), whereas in those treated with rose bengal, a progressive vacuolization of the cytoplasm was observed upon illumination. At 10–20 μM of dye the epimastigotes showed dispersion of the nuclear chromatin and a less electron-dense cytoplasm containing numerous vacuoles. No alterations in the kinetoplast-mitochondrion complex or in the kinetoplast-DNA could be observed in most of the cells under these conditions (Fig. 2b). Similar alterations were observed when trypomastigotes were used instead of epimastigotes (not shown). In addition, animals inoculated with rose bengal-treated trypomastigotes (20–100 μM) did not show parasitemia for at least 30 days. Controls infected with untreated parasites died at days 14–18 with high parasitemia (5×10^7 cells/ml).

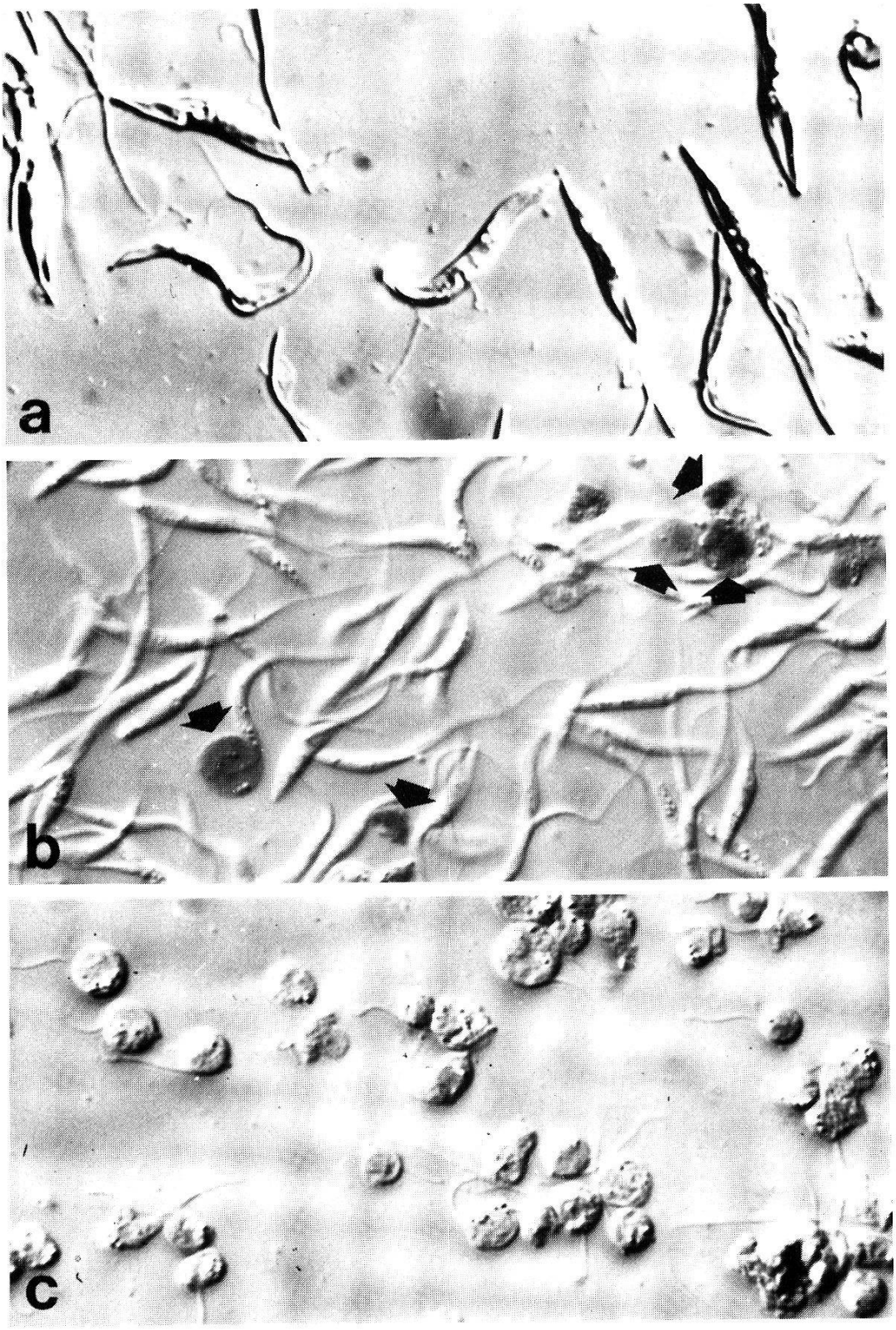

Fig. 1. *Trypanosoma cruzi* epimastigotes photographed using Nomarski differential interference contrast microscopy. (a) Normal cells in absence of rose bengal, ×1800. (b) After 60 min of preincubation in the presence of 20 μM rose bengal (in the dark) and exposed to the light of the microscope for 2 min, ×1500. (c) After 60 min of preincubation in the presence of 20 μM rose bengal (in the dark) and 10 min of incubation in the presence of light. ×1500. The arrows in (b) indicate the rounded cells. Other conditions as in "Materials and Methods" section.

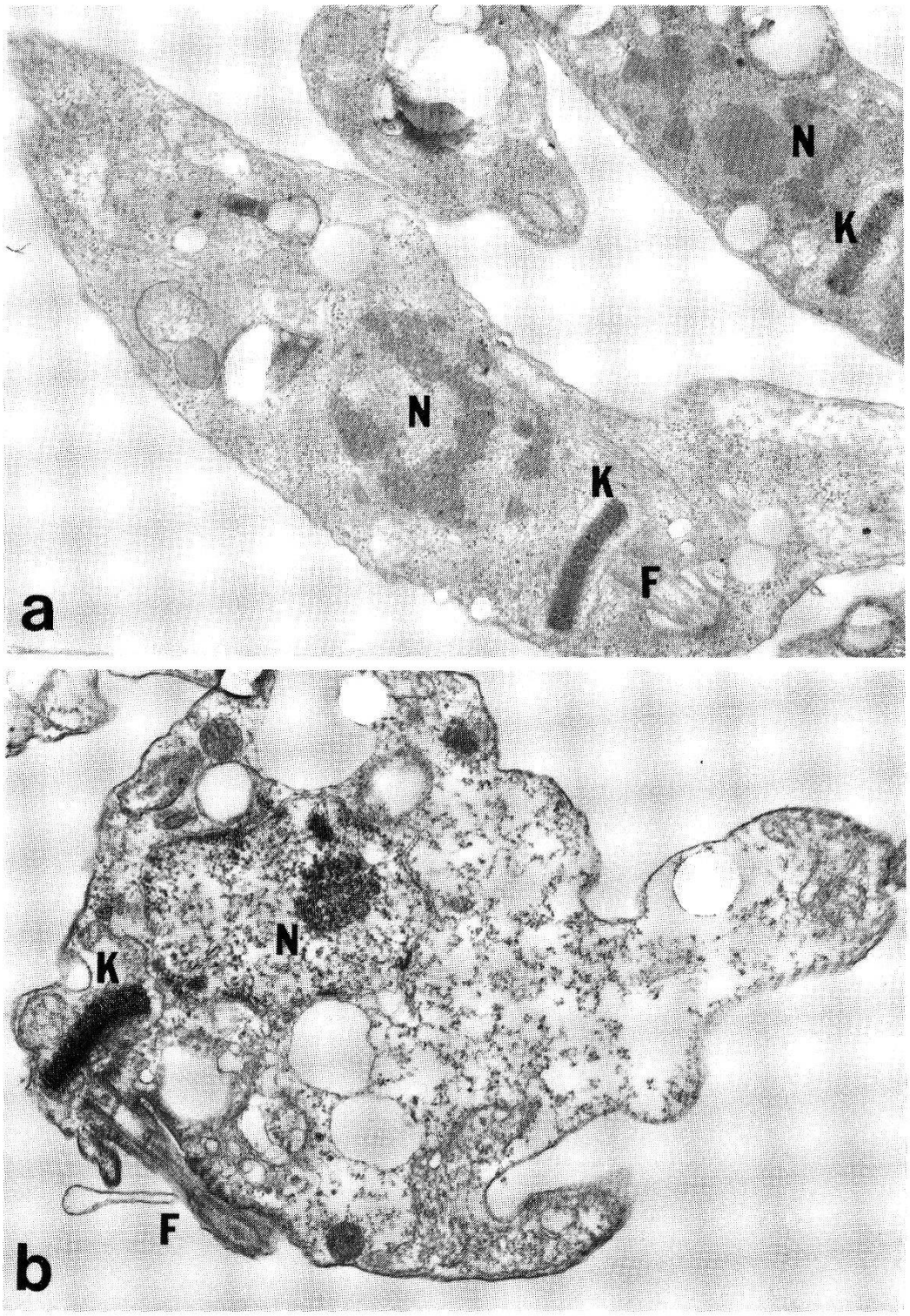

Fig. 2. Electron micrographs of *Trypanosoma cruzi*. (a) After 60 min of preincubation in the absence of rose bengal (in the dark) and 10 min of incubation in the presence of light (control), ×24,000. (b) After 60 min of preincubation in the presence of 20 μM rose bengal (in the dark) and 10 min of incubation in the presence of light, ×24,000. Abbreviations used: N = nucleus, K = kinetoplast, F = flagellum.

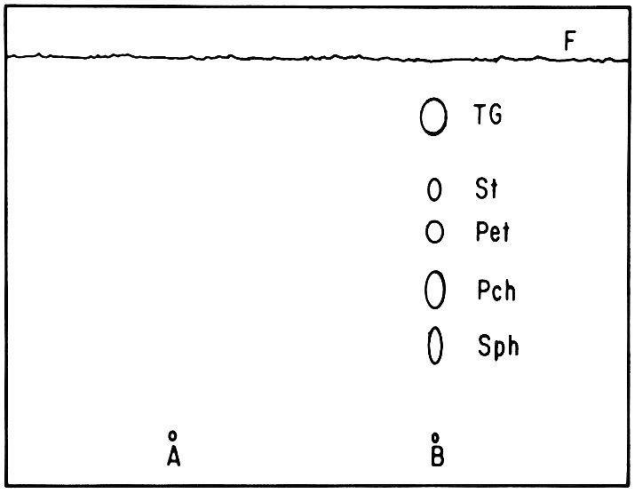

Fig. 3. Detection of lipid peroxides by thin-layer chromatography. *T. cruzi* epimastigotes were preincubated in the presence of 100 μM rose bengal for 60 min in the dark, and for 10 min under illumination as described in "Materials and Methods" section. Samples of cell suspensions were extracted and treated as described (Stahl, 1958). The chromatograms were developed by the starch-iodide procedure of Stahl (1958) to visualize the peroxides. (A) Control cells incubated in the absence of rose bengal. (B) Rose bengal-treated cells. Abbreviations used: Sph = sphingomyelin; Pch = phosphatidylcoline; Pet = phosphatidylethanolamine; St = sterols; TG = triacylglycerols.

Previous studies have shown that illumination of lipids in the presence of a rose bengal photosensitized-$^1O_2$-generating system yielded lipid peroxides (Kellog and Fridovich, 1975). In agreement with those results, illumination of *T. cruzi* epimastigotes preincubated for 60 min in the presence of 100 μM rose bengal yielded lipid peroxides. This effect was indicated by the appearance of spots corresponding to lipid peroxides on developed thin-layer chromatograms (Fig. 3).

When rose bengal (1 mM) was incubated under anaerobic conditions with intact *T. cruzi* epimastigotes (Fig. 4 A), a single-line ESR spectrum was detected (Leaver, 1971). Omission of *T. cruzi* cells or rose bengal led to a total lack of this signal (Fig. 4 C). The signal was not observed under aerobic conditions (not shown). Care was taken to keep the incubations in the dark, because, after exposure to room light or the light of a slide projector, the signal grew in intensity (Fig. 4 B). Changes in the radical concentration during illumination showed the dependence of the signal intensity on the illuminating light (Fig. 5). The kinetics of the ESR signal was characterized by repeatable exponential rise and decay curves with similar rates of radical decay in the presence or absence of light. The light-independent first-order decay constant was 0.77 per min ($t_{1/2}$ = 0.9 min).

Discussion

Sensitized photooxidations can proceed by two major mechanisms (Foote, 1976). In the so-called type I reaction, the excited sensitizer interacts with another molecule directly, usually with transfer of a hydrogen atom or an electron.

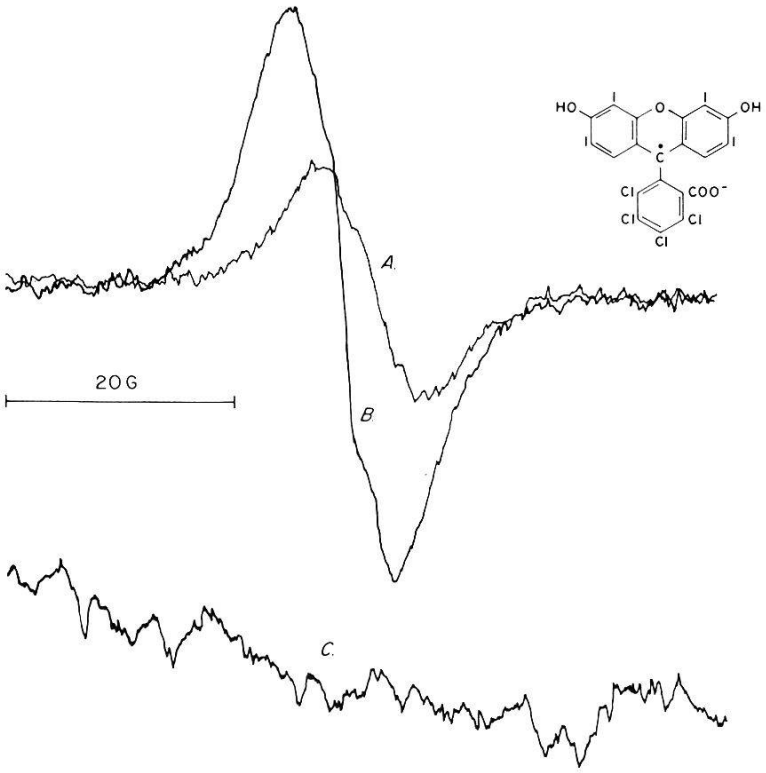

Fig. 4. Electron spin resonance spectra from incubations of *Trypanosoma cruzi* epimastigotes. (A) Spectrum from an incubation of epimastigotes (25 mg protein/ml) containing 1 mM rose bengal and PBS (pH 7.4). The oxygen was displaced by purging with nitrogen, and the reaction was initiated with rose bengal. The nominal microwave power was 20 mW, and the modulation amplitude was 3.5 G. (B) Spectrum obtained as in (A) after illuminating the flat cell with incandescent light from a slide projector for 2 min. (C) The same as in (A) but without rose bengal.

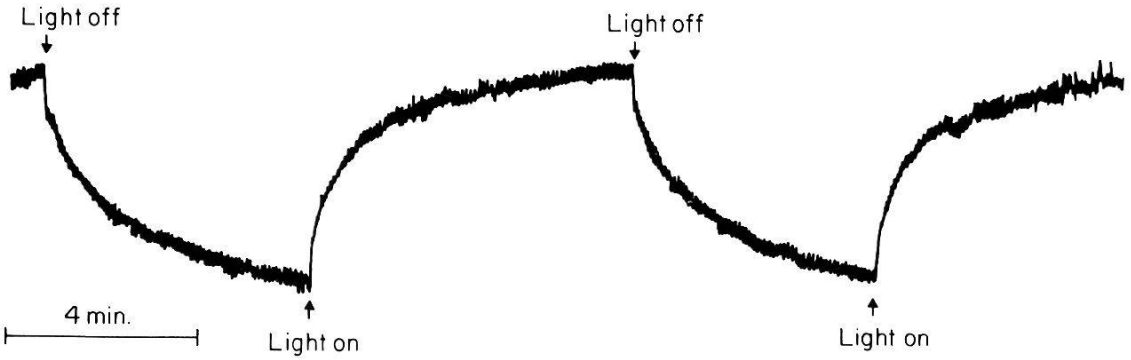

Fig. 5. Continuous record of the changes in the ESR signal amplitude of the rose bengal radical during illumination at the field position of maximum displacement of the derivative signal.

The radicals thus formed undergo further reaction with oxygen or other organic molecules. In the second class of reaction, called type II, the sensitizer triplet interacts with oxygen, most commonly by energy transfer, to give singlet oxygen, which reacts further with various acceptors in solution (Foote, 1976). Less efficiently, electron transfer from sensitizer to oxygen occurs, leading to superoxide anion and an oxidized form of the sensitizer (Foote, 1976). However, electron transfer occurs in less than 1 in 100 deactivating collisions of oxygen with most sensitizer triplets (Foote, 1976). In the presence of light and oxygen,

rose bengal is a good singlet oxygen producer (type II reaction) (Houba-Herin et al., 1982). Under these conditions rose bengal caused oxidative damage to *T. cruzi*. The production of lipid hydroperoxides was demonstrated by thin-layer chromatography, and it seems probable that membrane lipid peroxidation was important in the observed ultrastructural alterations. These changes are consistent with an increased permeability of the cells which led to gradual osmotic swelling and ultimately to lysis. As a result of this treatment the infectivity of trypomastigotes in mice was abolished.

In the absence of light and under anaerobic conditions rose bengal undergoes, in intact trypanosomes, a one-electron reduction to produce a carbon-centered free radical as demonstrated by ESR spectroscopy. This was the best evidence that the dye is incorporated into the cells. Moreover, the formation of this free radical may be involved in the motility inhibition observed in cells pre-incubated in the dark at high concentrations of rose bengal. In the presence of *T. cruzi*, visible light photoreduced rose bengal to the same carbon-centered radical that was formed by enzymatic reduction, thus implying that under anaerobic conditions a type I reaction is also possible.

The influence of light on the trypanocidal action of other drugs has been reported previously (Meshnick et al., 1978; Docampo et al., 1983). Light potentiates the lytic effect of hematoporphyrin and several related porphyrins on *Trypanosoma brucei* (Meshnick et al., 1978) and enhances the trypanocidal action of crystal violet on *T. cruzi* (Docampo et al., 1983). In addition, in agreement with our results with rose bengal, crystal violet undergoes one-electron reduction in intact trypanosomes under anaerobic conditions to produce a carbon-centered free radical as demonstrated by ESR spectroscopy. The formation of this free radical was also enhanced by light (Docampo et al., 1983). However, it has been demonstrated that, in contrast to rose bengal (Houba-Herin et al., 1982), the photolysis of crystal violet under aerobic conditions does not produce singlet oxygen (Gennari et al., 1974), but rather superoxide anion (Fischer et al., 1984). The difference noted between crystal violet's and rose bengal's capability to generate oxygen reduction or excitation products may be significant in the chemoprophylaxis of Chagas' disease when these dyes are compared for their toxicity to erythrocytes. Although crystal violet has been widely used in blood banks to prevent blood transmission of Chagas' disease without producing hemolysis (Nussenzweig et al., 1953; Brener, 1975), the hemolytic effect of rose bengal is well known (Blum and Kautzmann, 1953).

It has been postulated (Williamson, 1979) that the ultrastructural alterations determined by trypanocidal drugs may be of value in identifying their mode of action. In agreement with that assumption, treatment of *T. cruzi* with $\dot{O}_2^-$ and/or H_2O_2-generating drugs (i.e. naphthoquinones [Docampo et al., 1977, 1978a, 1978b; Cruz et al., 1978] or crystal violet [unpublished results]) or *T. dionisii* with H_2O_2 and myeloperoxidase (Thorne et al., 1981) determined similar ultrastructural alterations: swelling of the kinetoplast-mitochondrion

complex and condensation of the nuclear chromatin in patches (Docampo et al., 1977; Cruz et al., 1978; Thorne et al., 1981). In contrast, treatment of *T. cruzi* with rose bengal resulted in ultrastructural alterations clearly different from those mentioned before: vacuolization and swelling of the cytoplasm and loss of electron-density of the nucleus and the cytoplasm.

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