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Effect of ultraviolet and γ-radiations on *Herpetomonas samuelpessoai*

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Summary

A study was made about the influence of ultraviolet (UV) and γ-radiations on *Herpetomonas samuelpessoai* grown either in a chemically defined or in a complex medium. Cells cultivated in defined medium were more sensitive to UV than those from complex medium, as estimated by inhibition of cellular growth. The effect of γ-radiation, however, was independent of the media in which the cells were grown. Both radiations interfere with the plasma membrane as analysed by parameters such as excretion of cellular material and concanavalin-A-induced agglutination. Doses of UV which inhibit the cellular growth do not interfere with the plasma membrane. With γ-radiation, however, doses which inhibit cellular growth also interfere with the plasma membrane. These results suggest that for certain applications UV radiation may be an advantage in vaccine production.

Key words: Trypanosomatidae; *Herpetomonas samuelpessoai;* ultraviolet; gamma radiations; growth inhibition; effect on plasma membrane.

Introduction

Several evidences indicate that radiations affect properties of the cell membrane such as its structure and permeability (for a review see Myers, 1970). In a previous paper (Esteves et al., 1978) we showed that ultraviolet light affected the cell membrane of *Herpetomonas samuelpessoai* as evaluated by cell agglutination with the plant lectin concanavalin A (Con A). Since an alteration of the cell membrane may interfere with the excretion of cellular substances, ex-

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periments were carried out in order to determine whether cellular components are released at increased rates in UV- and γ-irradiated cells.

Herpetomonas samuelpessoai is a trypanosomatid which can be cultivated either in a complex or in a defined medium at 28 or 37° C. Hence, it can be used as a model to study the influence of growth conditions on cellular radiosensitivity. Indeed, previous studies from our laboratory have shown that the cell membrane of *H. samuelpessoai* changes according to the growth conditions (Sixel et al., 1978).

In this paper we describe results of a study with *H. samuelpessoai* which show that UV and γ-radiations damage the plasma membrane, as judged by interference in cell agglutination by Con A, and release of cellular components. The results also show that the cellular radiosensitivity estimated by parameters such as growth inhibition, Con A binding and excretion of cellular material varies according to the growth conditions of the cells.

Material and methods

Microorganism. – *Herpetomonas samuelpessoai* was maintained by weekly transfers in a chemically defined medium (Roitman et al., 1972) and distributed in 5 ml volumes in screwcapped tubes. Cells were grown at 28° C for 48 h. Cultures were kept at 4–6° C before use.

Mass culture. – *H. samuelpessoai* was grown at 28° C in 500 ml flasks with 250 ml chemically defined medium or complex medium with the following composition (g/l): sucrose 20; KCl 20; trypticase 4 (Baltimore Biological Laboratory, USA); yeast extract 3 (E. Merck, Germany); folic acid 0.02; hemin 0.02 (type II, Sigma Chemical Company, USA) dissolved in quadrol (J. T. Backer Chemical Company, USA) 25%, pH 7.0. The media were autoclaved at 120° C for 20 min. Inocula consisted of 5 ml of a 48-h culture in the defined medium. About 4.10^7 cells/ml were inoculated per flask. After 48 h cells were collected by centrifugation (2000 *g*) at 4° C for 10 min, were washed 4 times in cold phosphate-buffered saline (PBS), pH 7.2 and suspended in the same buffer to a concentration of 1.10^8 cells/ml or 2.10^8 cells/ml depending of the kind of the experiments.

Radiation sources. – The source of UV-light was a General Electric 15-W low pressure germicidal lamp with a maximum UV-energy output at a wave-length of 253.7 nm. Doses were measured with a photoelectric dosimeter (Latarjet et al., 1953).
The fluence rate measured at the level of a preparation was 3.3 $W.m^{-2}$. Irradiation was carried out in air at room temperature with gentle shaking.

Irradiation with γ-rays were performed at room temperature using an Irradiator Model GR-9 (U.S. Nuclear Corporation), containing cobalt-60 as a radiation source (dose rate, approximately 400 rads/min).

Effect of UV and γ-radiations on cell growth. – Since the efficiency of plating of *H. samuelpessoai* is low we could not estimate the number of cells by counting colonies as is usually employed in radiobiological studies of bacteria. Instead, we used the percentage of growth inhibition as the criterium to assay the effect of radiation.

The cells cultivated either in complex or defined media were rinsed in PBS and irradiated with UV or γ-radiations (with doses indicated in the figures) and then inoculated into either a complex or defined media. These experiments were made in 18×150 mm tubes containing 5 ml of defined or complex media and capped with stainless steel closures. Inocula per tube consisted of 0.1 ml from the cell suspension ($2 \cdot 10^8$ cells/ml) irradiated. Measurements of the growth were made at intervals of 24 h as absorbance (OD) at 540 nm. Results are expressed as percentage of growth inhibition and are the average of at least 2 determinations.

Effect of UV and γ-radiations on release of cellular components. – The cells cultivated either in

complex or defined media were washed in PBS and irradiated with UV or γ-radiations (with doses indicated in the figures). After irradiations the cell suspension ($1 \cdot 10^8$ cells/ml) was immediately centrifuged (2000 *g*) at 4° C for 10 min. The following analyses were made in the supernatants: peptide contents (Lowry et al., 1951), UV spectra (from 220 to 300 nm) and UV absorption at 260 nm. The UV measurements were taken in the Beckman Acta III spectrophotometer. In these experiments cellular lysis was controlled by counting the number of flagellates in the hematocimetric Neubauer chamber before and after UV and γ-irradiations.

Agglutination tests. – The flagellates cultivated in complex medium were irradiated with γ-radiation (doses in table and figures). After irradiation the cells were washed three times in PBS and incubated with Con A. Purified concanavalin A (grade IV, Sigma) solutions were freshly prepared before each experiment. The agglutination was carried out in glass tubes at 28° C for 30–60 min with occasional shaking. Equal volumes of the cell suspension (containing $2 \cdot 10^8$ cells/ml) and a 1000 μg/ml solution of Con A were mixed rapidly. A rapid slide agglutination test was used. The intensity of agglutination was scored from 0 to + + + + by visual examination of cells with a Zeiss Universal photomicroscope with a 16/0.35 phase contrast objective, to obtain a final magnification of 140× in a 6×9 cm plate film. The specificity of the agglutination reaction was controlled: a) by incubating cells in PBS without Con A to check any autoagglutination and b) by inhibiting the Con A reaction with α-methyl-D-mannoside (0.2 M).

Electron microscopy and cytochemistry. – Cells washed in PBS after γ-irradiation were collected by centrifugation, washed in a solution of 0.1 M Na-cacodylate buffer, pH 7,4 and were fixed in 2.5% (v/v) glutaraldehyde in the same buffer for 1 to 2 h at room temperature. The Con A-horseradish peroxidase-diaminobenzidine (Con A-HPR-DAB) method (Bernhard and Avrameas, 1971) for detection of Con A-binding sites was used. A detailed description of these methods is given elsewhere (De Souza et al., 1976). Control specimens consisted of: a) glutaraldehyde fixed cells exposed to horseradish peroxidase and diaminobenzidine without incubation with Con A; b) glutaraldehyde fixed cells which after incubation with Con A + α-methyl-D-mannoside (0.2 M), were treated with horseradish peroxidase and diaminobenzidine.

Results

Effect of UV and γ-radiations on cell growth

The inhibition of the growth was influenced by the dose of radiation and the growth conditions of the cells before irradiation. Fig. 1 shows that cells cultivated in a defined medium were more sensitive to UV than those cultivated in a complex medium. After 24 h cells originated from a defined medium show an inhibition of growth of about 80% for a UV dose of 45 Wm^{-2} while in those from complex medium the inhibition was of about 55%. This difference was also observed after 48, 72 or 96 h of cultivation (Fig. 1). After irradiation with a dose of 90 Wm^{-2} growth was observed only after 48 and 72 h for cells taken from complex and defined media respectively.

Experiments were also done in order to see if the nature of the medium used for cultivation of the cells after UV-irradiation influenced the radiosensitivity. Cells grown either in complex or defined media were irradiated and then inoculated in both media. Figs. 1 and 2 show that lower inhibition of growth was observed when the cells were inoculated in the complex medium.

With γ-radiation we did not observe differences in radiosensitivity between cells grown in complex or defined medium. The nature of the culture medium in which the cells were inoculated after irradiation also did not interfere with the

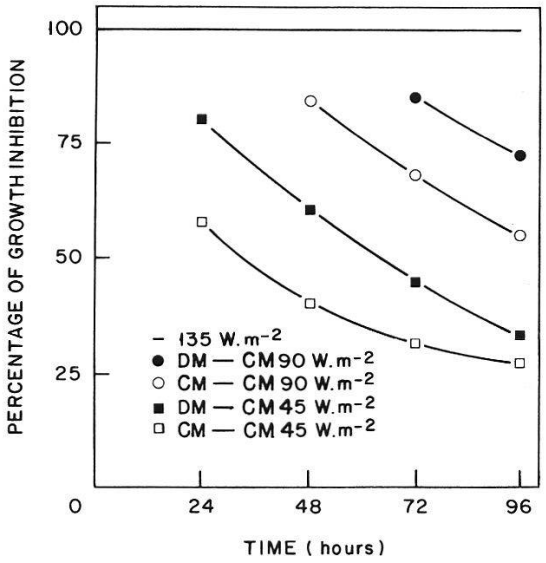

Fig. 1

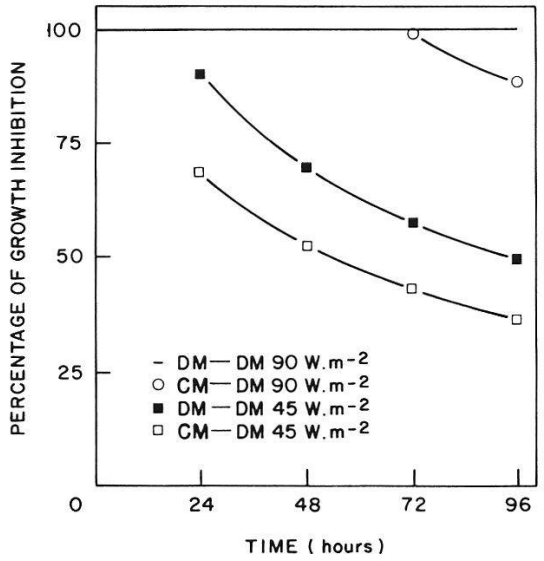

Fig. 2

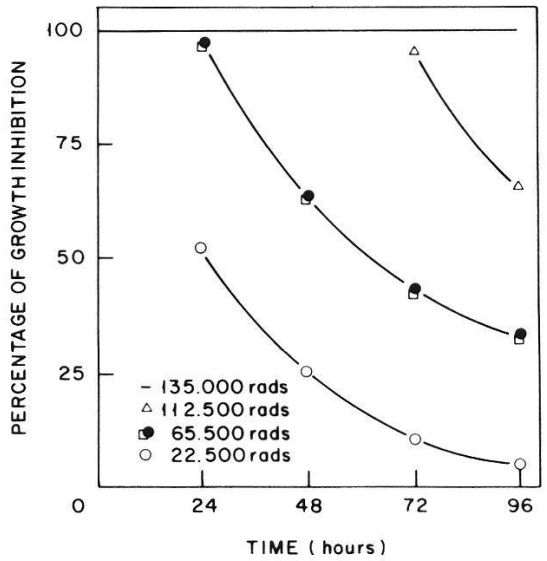

Fig. 3

Fig. 1. Effect of ultraviolet on the cellular growth of *H. samuelpessoai* grown either in defined (DM) or complex (CM) media and incubated after irradiation in complex medium.

Fig. 2. Effect of ultraviolet on the cellular growth of *H. samuelpessoai* grown either in defined (DM) or complex (CM) media and incubated after irradiation in defined medium.

Fig. 3. Effect of γ-radiation on the cellular growth of *H. samuelpessoai.*

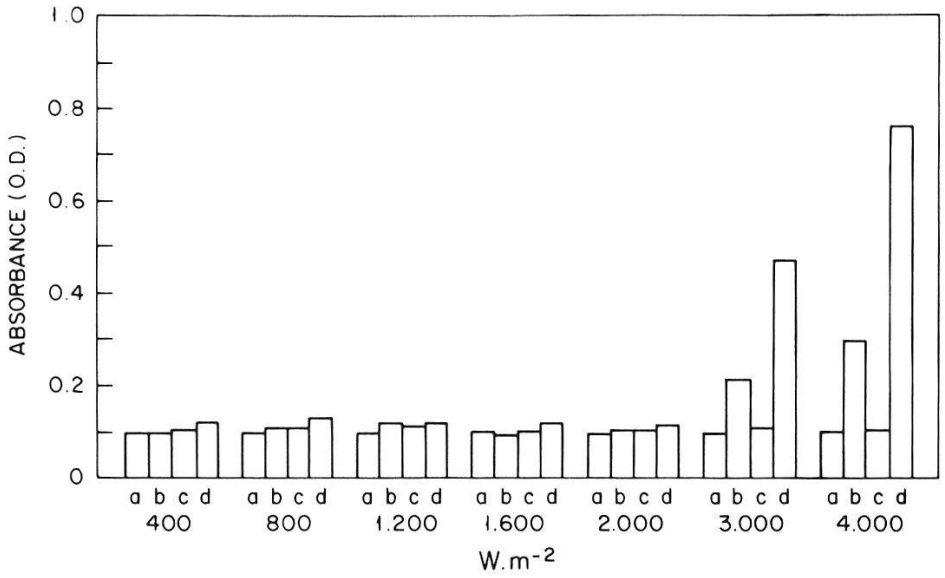

Fig. 4. Excretion of cellular material which absorbs in the UV at 260 nm by *H. samuelpessoai* derived either from complex or defined medium and irradiated with UV. a = non irradiated cells derived from complex medium; b = irradiated cells derived from complex medium; c = non irradiated cells derived from defined medium; d = irradiated cells derived from defined medium.

cellular growth. Fig. 3 shows the effect of γ-radiation on *H. samuelpessoai.* No growth was observed with doses higher than 112,500 rads.

Effect of UV and γ-radiation on the release of cellular constituents

Experiments were done in order to look for effect of radiation on release of cellular components through the plasma membrane. After irradiation the cell suspension was centrifuged and the supernatant was analysed. The UV spectra (from 220 to 300 nm) of the material released showed a peak of absorption at 260 nm. The amount of cellular components released varied according to the dose of UV radiation and with the origin of the cells. Cells cultivated in defined medium were more sensitive than those from complex medium. With a dose of 4000 Wm^{-2} we observed an increase in released material of about 3 and 5.5 times for cells cultivated in complex and defined media respectively (Fig. 4). Release of cellular materials was only observed with doses higher than 2000 Wm^{-2}.

No differences in excretion of products that absorb in the UV at 260 nm were found between control and γ-irradiated cells when doses of 22,500 or 45,000 rads were used. With higher doses, however, irradiated cells excreted less than control cells except for doses up to 112,500 rads when cells derived from defined medium showed high excretion (Fig. 5). In these experiments we used a cobalt source with a dose rate of 400 rads/min, so that in order to get the doses used the cells were irradiated for long periods. Since preliminary experiments had shown the existence of spontaneous excretion of cellular components we left control cells in buffer for the same periods of irradiation. For this reason the

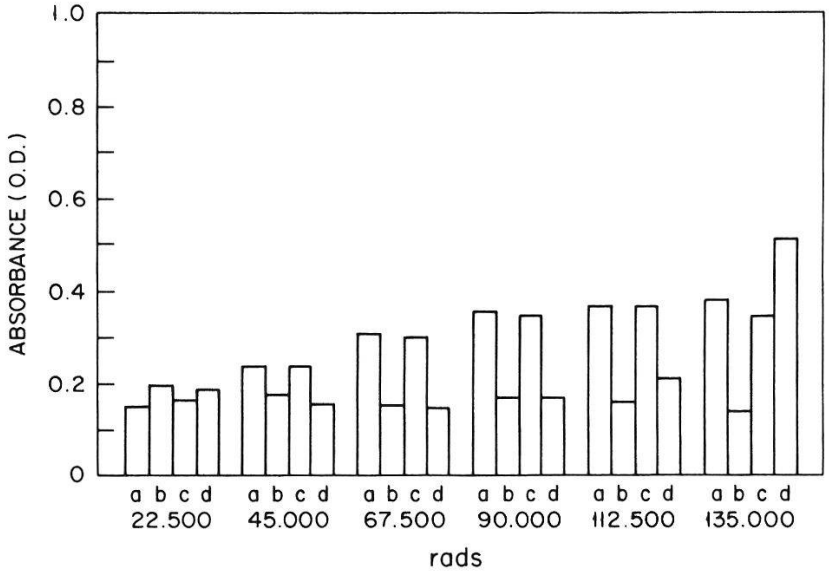

Fig. 5. Excretion of cellular material which absorbs in the UV at 260 nm by *H. samuelpessoai* derived either from a complex or defined medium and irradiated with γ-radiation. a = non irradiated cells derived from complex medium; b = irradiated cells derived from complex medium; c = non irradiated cells derived from defined medium; d = irradiated cells derived from defined medium.

Table 1. Effect of γ-radiation on the intensity of the agglutination of *H. samuelpessoai**

Dose (rads)	Intensity of agglutination
0	++++
22,500	++++
45,000	+++
67,500	+++/++
90,000	++
112,500	+
135,000	0

* Cells derived from complex medium. Concentration of concanavalin A: 500 μg/ml.

absorbance in control cells of experiments with γ-radiation is higher than that found in experiments with UV.

In general with both radiations the pattern of excretion of peptidic material was similar to that of release of the products that adsorb in the UV at 260 nm.

Effect of γ-radiation on the cell membrane binding of concanavalin A (Con A)

We showed in a previous report (Esteves et al., 1978) that UV radiation affects the agglutinability of *H. samuelpessoai* by the plant lectin Con A. However, the Con A-binding sites, as detected by ultrastructural cytochemistry, were not lost. We did similar experiments with γ-radiation and observed that it also interferes with *H. samuelpessoai* Con A-induced agglutination. The intensi-

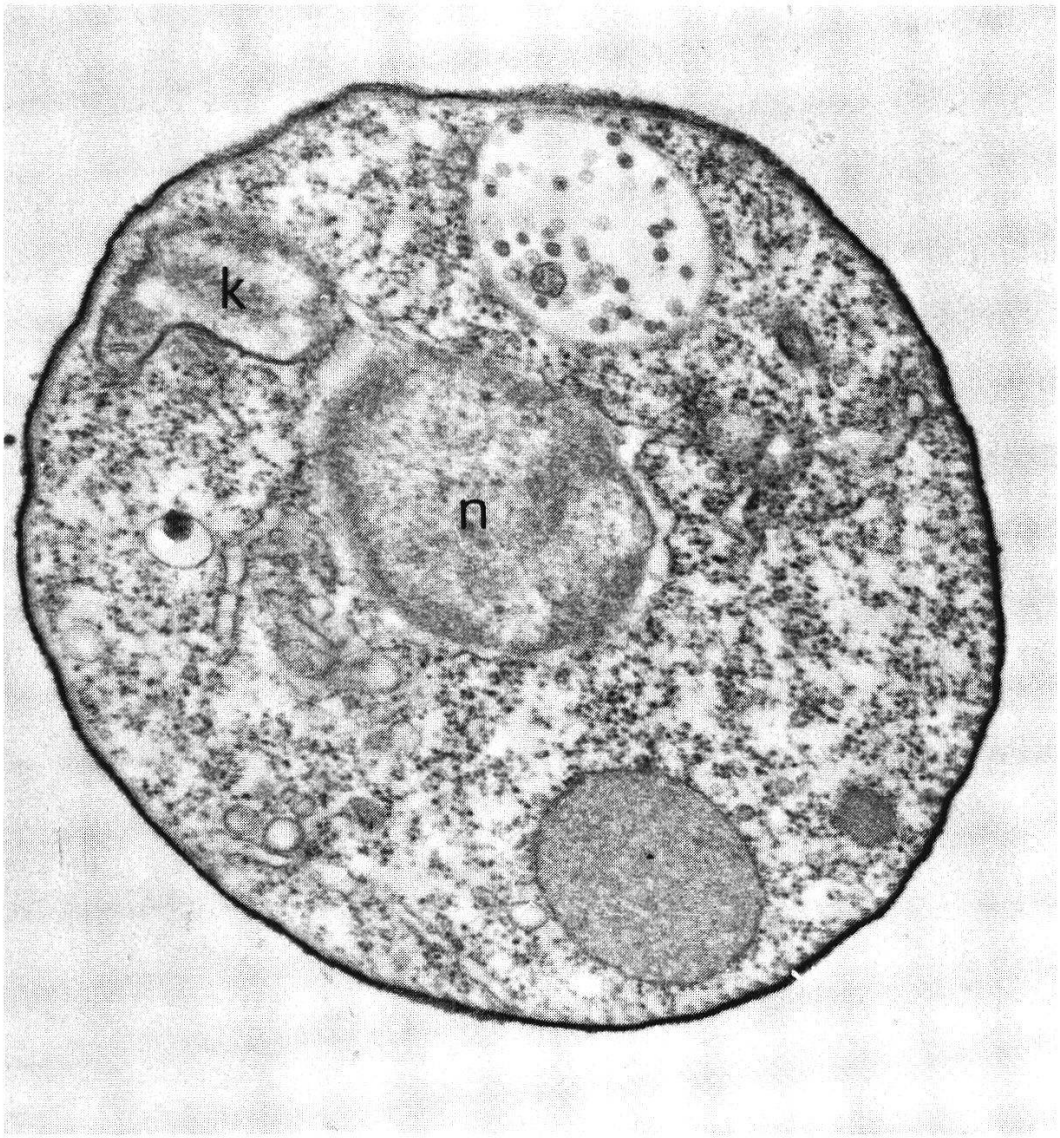

Fig. 6. *Herpetomonas samuelpessoai* fixed with glutaraldehyde after γ-radiation and submitted to the concanavalin A-horseradish peroxidase-diaminobenzidine method. Con A-binding sites are observed all over the cell surface. N = nucleus; K = kinetoplast. $\times$25,000.

ty of the agglutination was dependent on the dose of γ-radiation (Table 1). With doses higher than 112,500 rads we did not observe agglutination. As shown in previous papers (De Souza et al., 1976; Sixel et al., 1978), the agglutination was specifically induced by Con A, since in the absence of the lectin or in the presence of the specific inhibitor α-methyl-D-mannoside, agglutination was not observed. To determine if irradiated cells, unable to agglutinate with Con A, possess Con A-binding sites on the plasma membrane, we have used the Con A-horseradish peroxidase-diaminobenzidine technique in association with electron microscopy. After irradiation with 135,000 rads cells exhibit a dense reactive layer on the plasma membrane (Fig. 6). The distribution of Con A-binding sites in these cells appeared similar to that seen in unirradiated cells. No reactive layer was observed on the plasma membrane of cells incubated with Con A

plus α-methyl-D-mannoside thus eliminating the possibility of non-specific binding of the horseradish peroxidase to the cell surface.

It should be stressed that even with highest doses of UV and γ-radiations used in all experiments the cells were not lysed and remained motile.

Discussion

We observed that differences in growth conditions affect the radiosensitivity of the protozoa. Cells grown in a complex medium are more resistant to UV radiation than cells grown in a defined medium. Differences in radio-sensitivity of *Escherichia coli*, as consequence of growth conditions, have been reported (Alper and Gillies, 1960). Contrary to what we observed with *H. samuelpessoai*, *E. coli* grown in a complex medium are more sensitive than those grown in defined medium. In complex medium *E. coli* has a high metabolic activity and a shorter generation time which could increase the radio-sensitivity. Differences in growth conditions did not change the generation time of *H. samuelpessoai*. It is possible that the differences in radiosensitivity observed with *H. samuelpessoai* are the consequence of growth-medium dependent biochemical differences.

Previous studies show that grown in defined medium *H. samuelpessoai* only synthesize ergosterol (Fagundes, 1974). However, in complex medium other esterols which absorb at 253.7 nm are also synthesized (Fagundes, 1974). It has been considered that the presence of esterols absorbing at 253.7 nm is a factor in the radioresistance. Growth conditions did not influence the sensitivity of *H. samuelpessoai* to γ-radiation. This result can be explained by the fact that while γ-radiation interacts randomly with biological macromolecules the UV radiation is absorbed mainly by molecules with alternated double bonds.

Differences in the cell surface of *H. samuelpessoai* grown in different conditions have been observed previously (Sixel et al., 1978). Cells grown in complex medium agglutinated strongly with the plant lectin concanavalin A and have concanavalin-A binding sites homogenously distributed throughout the cell surface. Cells grown in defined medium, however, agglutinated only by the flagellum and the concanavalin A binding sites are preferentially located on the flagellar membrane.

Although more emphasis has been given in recent years to the study of the effect of radiation on DNA (for a review see Giese, 1967) several evidences indicate that cell surface properties such as distribution of membrane components, membrane permeability, etc. are also affected by radiation (Myers, 1970; Koteles et al., 1976; Facchini et al., 1976; Sato et al., 1977). In a previous paper we showed that UV radiation interferes with agglutination of *H. samuelpessoai* by concanavalin A. While unirradiated cells agglutinate intensely with Con A, UV-irradiated cell did not. However, the Con A-binding sites were detected by electron microscopy, in both unirradiated or irradiated cells, sug-

gesting that UV-radiation interferes with the mobility of Con A-receptors in the plane of the plasma membrane (Esteves et al., 1978). Similar results were also obtained with γ-radiation as described in this paper. Modifications of the plasma membrane as a consequence of ionizing radiations, have recently been reported for erythrocytes (Sato et al., 1977), fibroblasts (Koteles et al., 1976) and lymphocytes (Facchini et al., 1976). Since the results obtained with *H. samuelpessoai* are evidence for alterations in the plasma membrane we decided to search for release of cellular components in UV- and γ-irradiated cells. Our results show that irradiated cells released peptides, and 260 nm absorbing material. The release of these materials is dependent on the dose of radiation. We also observed that the growth conditions of *H. samuelpessoai* affects the sensitivity to the UV radiation, as detected by release of cellular components. The release was more accentuated in cells grown in defined medium. Studies on other cells show that radiation interferes with the cell permeability for substances such as ATP (Billen et al., 1953), Na^+ and K^+ (Giese, 1967), amino acids, nucleotides (Billen, 1957), nuclease (Gentner and Mitchel, 1975), and polysaccharides (Mitchel, 1976).

Ionizing radiations have been used to obtain inactivated microorganisms to be used as vaccines (Duxbury and Sadum, 1969; Sech and Hucal, 1975; Kramer and Vanderberg, 1975; Eisenberg and Osterman, 1977). Since radiation affects cell surface components, which are antigenic, care should be taken in order to avoid changes in the antigenic properties of such components. We observed that only doses of UV higher than those which inhibit the cellular growth of *H. samuelpessoai*, interfere with the cell surface as analysed by Con A-induced agglutination and excretion of cell compounds. With γ-radiation, however, doses which interfere with the cell membrane also inhibit cellular growth. These results suggest that for certain applications UV radiation may be an advantage in vaccine production.

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