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Title: A COMPARISON OF RADIATION INDUCED LIPID CHANGES IN MICROCOCCUS RADIODURANS, SARCINA LUTEA, AND PSEUDOMONAS AERUGINOSA

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Quantity variations in pigments and lipid classes were determined following γ-irradiation of Micrococcus radiodurans, Sarcina lutea, and Pseudomonas aeruginosa.

Pigmentation was determined by extraction with organic solvents and measured as optical density at 475 mμ for M. radiodurans and S. lutea, and 410 mμ for Ps. aeruginosa. Pigments of M. radiodurans and S. lutea were inactivated at a linear rate with increased doses. The non-carotenoid pigment of Ps. aeruginosa was not significantly affected at a maximum dose of 2,040 krads.

Quantitation of neutral lipids and neutral lipid hydrolysates was accomplished with thin layer chromatography. The lipid classes involved were separated on thin layer plates, charred with concentrated H_2SO_4 and heating, and densitometric determinations made.

No differences were observed in the lipid classes of M. radiodurans and S. lutea following doses up to 2,040 krads. A dose of 2,040 krads resulted in a large increase in free fatty acids of Ps. aeruginosa.

A Comparison of Radiation Induced Lipid Changes in *Micrococcus radiodurans*, *Sarcina lutea*, and *Pseudomonas aeruginosa*

by

Ronald Douglas Ley

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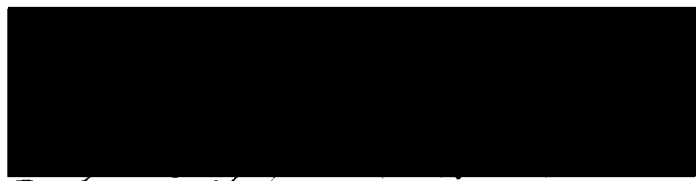

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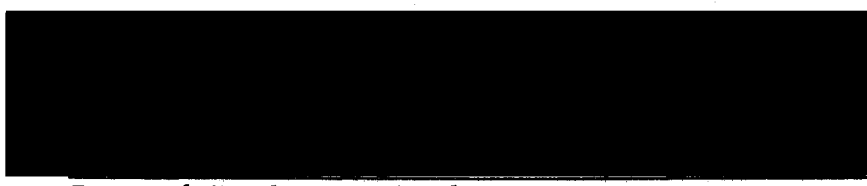

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TABLE OF CONTENTS

LIST OF FIGURES

A COMPARISON OF RADIATION INDUCED LIPID CHANGES IN MICROCOCCUS RADIODURANS, SARCINA LUTEA, AND PSEUDOMONAS AERUGINOSA

INTRODUCTION

Previous investigations of the radiation resistance of Micrococcus radiodurans have implicated certain lipid classes as contributing to this organism's ability to survive high doses of ionizing radiation.

In the past, the approach to the role of lipids as a radioprotective mechanism of M. radiodurans has primarily been concerned with the alteration of the quantity of these lipids in the cell, and subsequent correlation to an increase or decrease in radiation resistance. These lipid alterations were brought about by induced mutations (23), metabolic inhibitors (20), or nutrient modification of the growth medium (15). This approach has met with varying degrees of success and considerable contradiction.

This investigation was undertaken to measure the immediate effect of γ-radiation on lipid classes of M. radiodurans, S. lutea, and Ps. aeruginosa.

Quantitative measurement of the direct or indirect action of γ-radiation on the lipids considered was based on the assumption that the action sufficiently altered the chemical structure of the lipid to the extent that the product had non-lipid properties, the

migration of the molecule during thin layer chromatography was changed, or the absorption by a compound (carotenoids) at a specific wavelength was decreased.

Micrococcus radiodurans, S. lutea, and Ps. aeruginosa represent high, intermediate, and low microbial resistance to ionizing radiation, respectively.

LITERATURE REVIEW

Previous Investigation on the Lipids of M. radiodurans

The carotenoid pigments (14, 15, 23) and unsaturated fatty acids (18, 20) of M. radiodurans have been suggested as functioning as radioprotective compounds in this highly radiation resistant organism. The unsaturated lipid molecule presumably reacts with free radicals formed by ionizing radiation, thus preventing the free radical from damaging essential sites in the cell.

Krabbenhoft (15) caused a ten-fold decrease in the radiation resistance of M. radiodurans by the addition of NZ-Case (a tryptic digest of casein) to the growth medium. The decrease in resistance was shown to be accompanied by a marked decrease in the carotenoid pigments. The pigments of M. radiodurans had previously been identified as being carotenoids (17). Thin layer chromatography of the pigments from cells grown in the presence of NZ-Case showed a loss of two pigment components (designated by Krabbenhoft as pigments one and four of eight pigments) normally found in the more resistant cells grown in the absence of NZ-Case.

Moseley (23) reported isolation of mutants of M. radiodurans with decreased pigment content of the cells and decreased resistance to X-irradiation. One mutant with no pigmentation did not show the

characteristic "shoulder" on the sigmoidal survival curve of the M. radiodurans wildtype. He concluded that carotenoid pigments or carotenoid precursors contribute considerably to the resistance of M. radiodurans.

Moseley, et al. (24) later excluded the role of pigments in energy transfer reactions as a mechanism of radiation resistance in M. radiodurans, and reported that a DNA repair mechanism was responsible for the resistance.

Lewis (18) characterized the fatty acids of M. radiodurans and, upon finding several long chain polyunsaturated fatty acids, proposed that unsaturated fatty acids could possibly contribute to the organism's resistance to γ-radiation.

One investigator used diphenylamine in the growth medium to alter the lipid composition of M. radiodurans and reduce the organism's LD_{50} for X-rays from 213 kR to 134 kR (20). Upon examination of the lipid constituents following the diphenylamine treatment, he concluded that unsaturated lipids and fatty acids of M. radiodurans may act as radioprotective compounds.

A radioprotective agent has been demonstrated in M. radiodurans by Bruce (6). The structure of the compound has not been elucidated, but it is presumed to be of a sulfhydryl nature based on radiosensitization studies with p-hydroxymercuribenzoate (HMB) (7). N-ethylmaleimide, HMB, p-chloromercuribenzoate, iodoacetic acid,

and idoacetamide have been used to sensitize microorganisms to ionizing radiation (4, 5, 7, 9, 19, 25). This sensitization is thought to be due to binding with intracellular sulfhydryl protective agents (7, 19).

The only attempts to measure the effect of radiation on the lipids of M. radiodurans have involved the "bleaching" of the carotenoid pigments during irradiation. Krabbenhoft (15) reported the disappearance of five out of eight pigment components following exposure of M. radiodurans to a dose of γ-radiation lethal for 99.9 percent of the population. Two of the pigments which disappeared were the aforementioned pigments one and four.

Kilburn et al. (14) measured the linear decrease in total pigment activity (absorption at 475 mμ) at increasing dose levels. The radiosensitivity of all the pigment molecules was assumed to be equal. They concluded that a majority of the pigment molecules are not inactivated by energy transfer, but that a small fraction of the carotenoids or non-pigmented precursors of the carotenoids may function as energy sinks.

Enhanced bleaching of pigments was observed by Lee (16) when the cells were irradiated in the presence of iodoacetic acid. He postulated that the radiolytic products of iodoacetic acid were responsible for the increased destruction of the pigments by irradiation. This indicated that the action of iodoacetic acid as a sensitizing agent is

not confined to action on the sulfhydryl groups.

The Effect of Ionizing Radiation on Lipids

The work done on the effects of ionizing radiation on nucleic acids and proteins far exceeds that done on lipids and lipid-containing material. In addition, the majority of the lipid investigations have been confined to non-biological systems.

One reaction induced by the irradiation of lipid material is thought to be similar to the autoxidation of fats (13, 21).

Schauenstein (26) isolated autoxidation products of polyunsaturated esters and fatty acids emulsified in an aqueous phase. He reported that the first major reaction of lipid autoxidation in an aqueous phase is the formation of unstable hydroperoxides which react readily to form aldehydes, ketones, dimers, polymers, and epoxides. Some of these products were shown in this same study to inhibit glycolysis and respiration of tumor cells.

It is conceivable that irradiation induced peroxidation could also produce these products. γ-irradiation of oleic acid and methyl oleate resulted in a marked increase in peroxides and carbonyl compounds at doses of 33 R/sec for 17.1 hours (10).

Figure 1 depicts possible reactions leading to the formation of organic peroxides and carbonyl compounds following irradiation of lipids in an aqueous environment (2, pp. 116, 138, 148; 8, p. 5; 26).

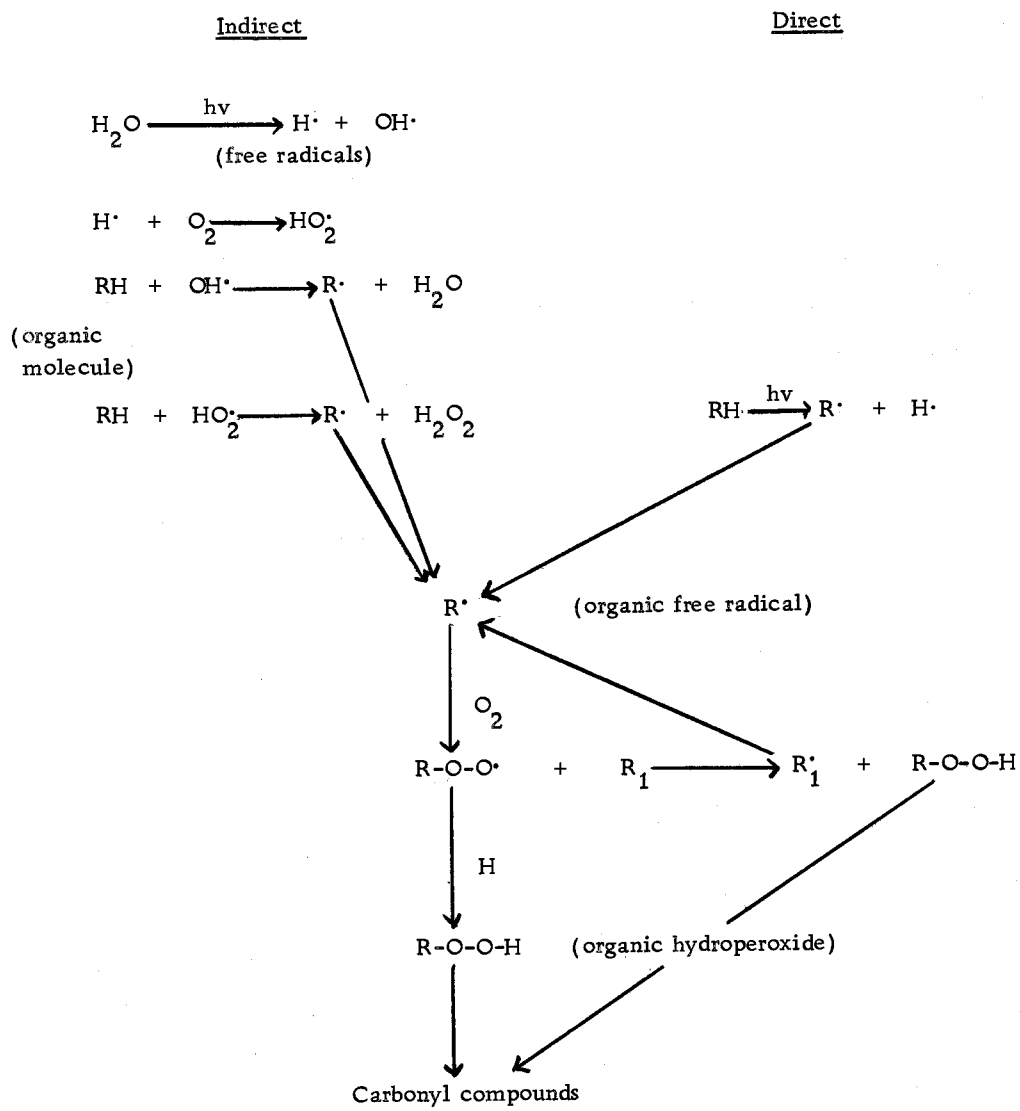

Figure 1. Possible reactions leading to the formation of organic peroxides and carbonyl compounds by the direct and indirect action of ionizing radiation.

The formation of organic peroxides within a living cell may have far-reaching biological effects, as organic peroxides have been shown to have mutagenic properties and to be extremely toxic to mice (2, p. 235).

Ionizing radiation has also been demonstrated as causing in vitro decarboxylation of fatty acids (3), and has increased the fatty acid content of irradiated cottonseed oil (1).

The Enzyme Release Hypothesis

The effect of ionizing radiation on the lipid constituents of a cell is of interest when considering the enzyme release hypothesis (2, p. 272-279) as a possible cause of cell death following irradiation. According to enzyme release hypothesis, cell death results from a primary lesion which alters the permeability of cellular and intracellular membranes. The altered permeability results in the disorganization of enzyme systems in the cell and leakage of material from the cell.

Increased membrane permeability to enzymes and cations following irradiation has been reported (2, p. 274; 12, p. 34-37; 22). Thus, the presence of lipids as a major component of cell membranes becomes important when considering alteration of permeability following irradiation.

Resistance of the Test Organisms to Ionizing Radiation

Bacteria, as well as other unicellular organisms, are considerably more resistant to radiation than multicellular organisms.

The pseudomonads are noted as being radiosensitive bacteria. A LD_{90} of 5-10 krads has been reported for this group (5).

Sarcina lutea represents median radiation resistance for bacteria with a LD_{90} of 60± krads (7).

Micrococcus radiodurans is one of the most radioresistant organisms known. A LD_{90} of > 400 krads, depending on culture conditions, has been reported for this organism (7).

MATERIALS AND METHODS

Microorganisms

The cultures of M. radiodurans, S. lutea, and Ps. aeruginosa were obtained from the stock culture collection at Oregon State University. Each organism was treated comparably throughout the study.

Culture Methods

The culture medium used for this investigation was a tryptone-glucose-yeast extract-methionine (TGYM) medium which consisted of:

Tryptone (Difco)	5.0 g
Yeast extract (Difco)	1.0 g
Glucose	1.0 g
DL-Methionine	20 mg
Distilled H_2O	1 liter

The pH of the medium was adjusted to 7.0, and the medium was distributed in 100 ml quantities into 250 ml DeLong culture flasks prior to sterilization. Each flask was inoculated with 1.0 ml of a 24 hour TGYM broth culture and incubated for 44 hours at 30 C on a gyrotory shaker.

Cell Harvest

The cells were collected by centrifugation at 7,000 × G for 15 minutes and washed twice with sterile 0.1% tryptone (Difco) solution. The wet packed cells were resuspended for irradiation in the same tryptone solution in 1:1 (w/v) proportions.

Irradiation

Ten ml aliquots of the washed, resuspended cells were placed in sterile irradiation vials and irradiated in the Cobalt-60 source at Oregon State University at room temperature. The dose rate was 510 krads per hour. Following irradiation, the cell suspensions were washed twice with distilled water and lyophilized for 24 hours and stored in a desiccator until needed.

Lipid Extraction

Reagents. All chemicals used were of reagent grade, and solvents were redistilled prior to use.

Procedure. One-half gram (0.50 g) of lyophilized cells was suspended in 25 ml of chloroform-methanol (2:1, v/v) and extracted by refluxing for one hour under a nitrogen atmosphere. The suspension was filtered through a sintered glass funnel (medium grade), and the filtrate evaporated to dryness at 45 C under a stream of nitrogen.

The lipid residue was taken up in chloroform-methanol (2:1, v/v) and treated to remove non-lipid contaminants according to the method of Folch et al. (11). The resulting lipid preparation from each dose level was divided into two equal portions for pigment and lipid class quantitation.

Pigment Quantitation

The pigmentation of controls and irradiated cells of S. lutea and Ps. aeruginosa was recorded as total pigmentation and determined by measuring the optical density of the lipid extract taken up in chloroform-methanol (2:1, v/v). Optical densities were determined with a Bausch and Lomb Spectronic 20 at 475 mμ for S. lutea and 410 mμ for Ps. aeruginosa. The pigments of M. radiodurans were quantitated as individual components following separation by thin layer chromatography (TLC).

TLC of Pigments of M. radiodurans

Thin layer plates (20 × 20 cm) were prepared with Silica Gel G (E. Merck, A. G., Darmstadt, Germany) according to the method of Stahl (27). The portion of the lipid extract appropriated for pigment quantitation was suspended in 0.5 ml of chloroform-methanol (2:1, v/v) for application to the activated Silica Gel G layers. The extract was applied with a micropipette at a level of 30 μl per lane

for a total of six lanes.

The chromatogram was developed in benzene-methanol-acetic acid (87:11:2, v/v/v) using an S-chamber system and tank development chambers. The chromatogram was first placed in the S-chamber, and the solvent allowed to ascend approximately seven cm above the point of sample application. At this time, the chromatogram was removed from the S-chamber and quickly transferred to the tank chamber and the solvent front allowed to advance an additional eight cm before the development was terminated.

The pigment components were removed from the thin layer plate with a vacuum zone collector and eluted off the gel with chloroform-methanol (1:1, v/v). The entire procedure from spotting of the chromatogram to pigment elution was carried out in a "glove" box which had been preflushed with nitrogen to prevent oxidation of the labile pigments. The eluted pigment was diluted to three ml with the eluting solvent and the optical density determined at 475 mμ.

Quantitation of Classes of Neutral Lipids and Hydrolysis Products of Neutral Lipids

Thin Layer Chromatography

The thin layer plates were the same as those used for pigment separation.

The lipid preparation for lipid class quantitation was suspended

in 0.5 ml of chloroform:methanol (2:1, v/v) and applied to the Silica Gel G layers with a micropipette at levels of 30 μl, 15 μl, and 30 μl for M. radiodurans, Ps. aeruginosa, and S. lutea, respectively.

The chromatogram was developed to a height of 12 cm in hexane-diethyl ether-acetic acid (88:12:1, v/v/v) in a tank chamber. Stahl (27) reported that mixtures of these reagents are useful for separation of neutral lipids and hydrolysis products of neutral lipids into classes.

Densitometry

The developed chromatogram was uniformly sprayed with concentrated H_2SO_4 and charred at 200 C for 20 minutes. The lipid classes were present as dark spots against the white background of the thin layer plate. A glass plate (20 × 20 cm) was placed on the surface of the gel and the chromatogram scanned with a densitometer (Photovolt Corp., New York, N.Y., Model 542) equipped with a synchronous motor driven chromatogram stage (Model 52-C). The collimating slit assembly had a 0.1 × 6 mm aperture. The area under the light transmission curve was calculated for each lipid class.

To express variations in lipid class quantities following irradiation, the curve area from the irradiated lipid class was divided by the curve area from the unirradiated lipid class. This value was designated as the A_i/A_u ratio for a specific lipid class following irradiation.

RESULTS AND DISCUSSION

The inactivation of pigments of M. radiodurans in response to increased radiation doses is delineated in Figures 2 and 3. A linear dose-response was obtained; with each pigment exhibiting similar inactivation rates. A G value of 50 to 60 pigment molecules being inactivated per 100 ev absorbed by active pigment molecules has been previously calculated (14). The similar pigment inactivation curves dispute the previously proposed (14, 15, 20) possibility of one or more pigment components being more reactive in energy transfer reactions than the remainder of the pigments.

The pigment(s) of S. lutea was inactivated by irradiation at a rate corresponding to that of M. radiodurans. Figure 4 represents the decrease in total pigment content of S. lutea with increased dosage.

The chloroform-soluble pigment pyocyanin of Ps. aeruginosa did not show a significant decrease in optical density following in vivo irradiation at 2,040 krads. Pyocyanin is a non-carotenoid pigment which absorbs at 410 mμ.

Quantities of lipid classes of M. radiodurans and S. lutea showed negligible variation following doses up to 2,040 krads. The A_i/A_u ratios for the classes considered in both M. radiodurans and S. lutea were 1 ± 0.1 which is within the ten percent variability of

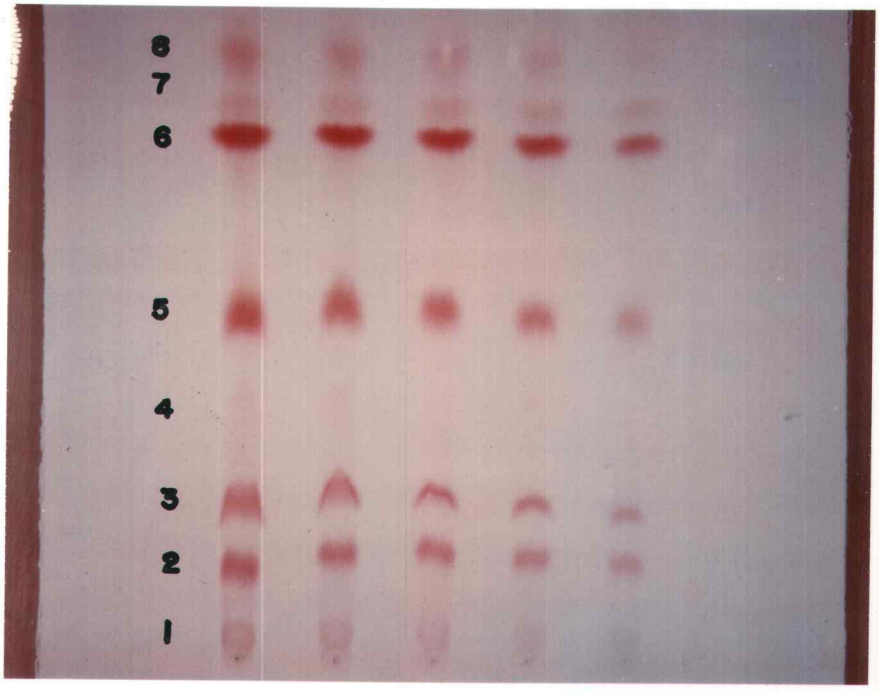

Figure 2. Thin layer chromatogram of pigments of M. radiodurans. Solvent system: benzene-methanol-acetic acid (87:11:2, v/v/v). The chromatogram was developed using an S-chamber system and tank development chambers. Doses (left to right): control, 255 krads, 765 krads, 1,275 krads, and 1,785 krads.

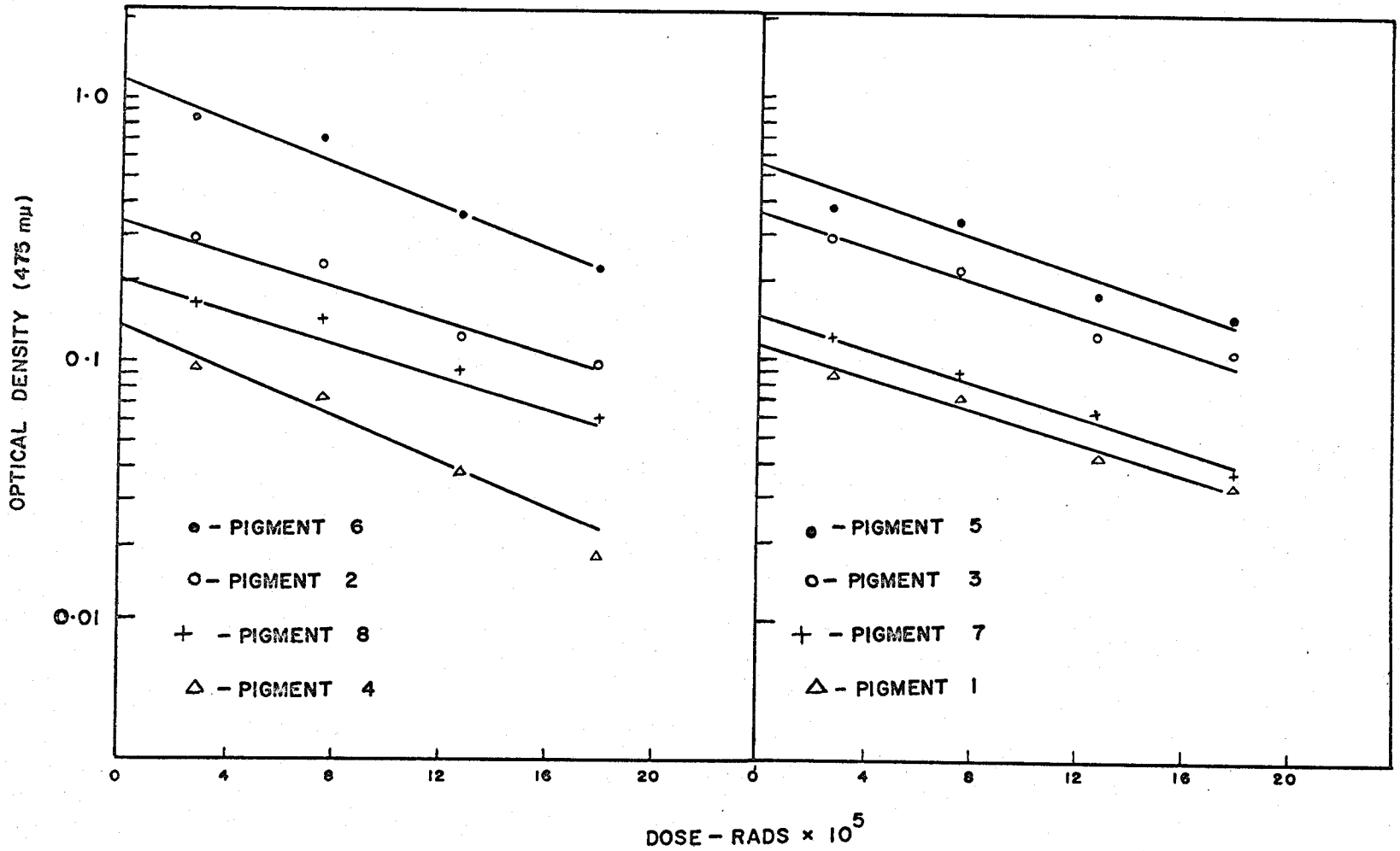

Figure 3. Inactivation curves of the carotenoid pigments of M. radiodurans. The pigments were separated as described for Figure 2, eluted from the gel, and the optical densities determined with a Bausch and Lomb Spectronic 20.

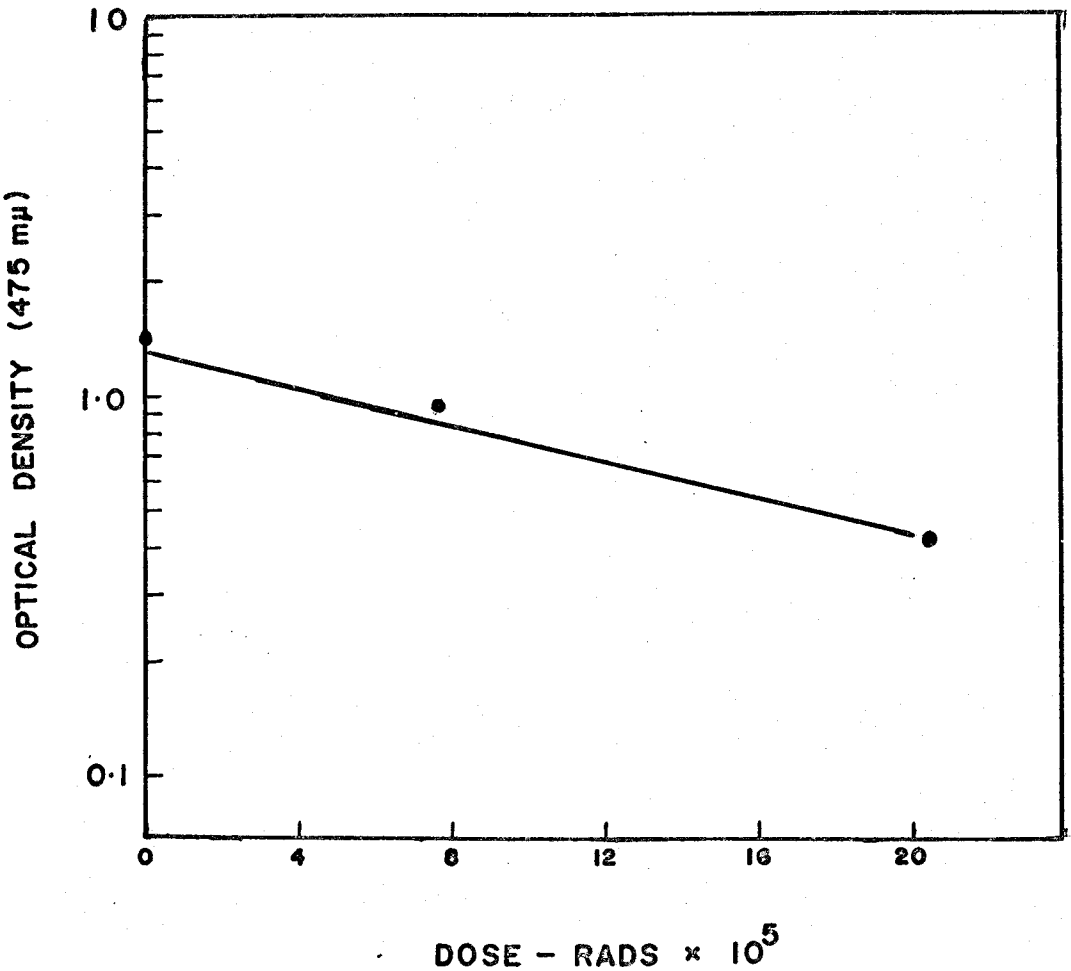

Figure 4. Inactivation curve of the total pigments of S. lutea. The pigment extract was taken up in chloroform-methanol (2:1, v/v) and the optical density determined with a Bausch and Lomb Spectronic 20.

of the method used to determine lipid class quantities.

Of the lipid classes considered, classes 1 and 4 of M. radiodurans (Figure 5) and classes 1 and 3 of S. lutea (Figure 6) are of particular interest. Lipid class 1 of M. radiodurans and S. lutea was tentatively identified as free fatty acids based on its relative position on the thin layer chromatogram. The lack of change in the quantity of free fatty acids would seem to indicate a noninvolvement of this lipid class in the radiation resistance of M. radiodurans as previously proposed.

Lipid class 4 of M. radiodurans and lipid class 3 of S. Lutea are considered carotenoid precursors based on TLC migration, color reactions, and light absorption maxima. No variation in this lipid class discounts the role of carotenoid precursors as a free radical "scavenger." Reaction with a free radical would undoubtedly result in a major alteration (unstable peroxide formation?) of the precursor molecule.

Irradiation of Ps. aeruginosa at a dose of 2,040 krads resulted in a large increase in free fatty acids. The increase in free fatty acids is illustrated in Figures 7 and 8. The A_i/A_u ratio for this lipid class was calculated as being approximately five.

This data indicates that the presence of carotenoid pigments may protect other lipid components of S. lutea and M. radiodurans. Protection of essential lipids in the cell could serve to maintain the

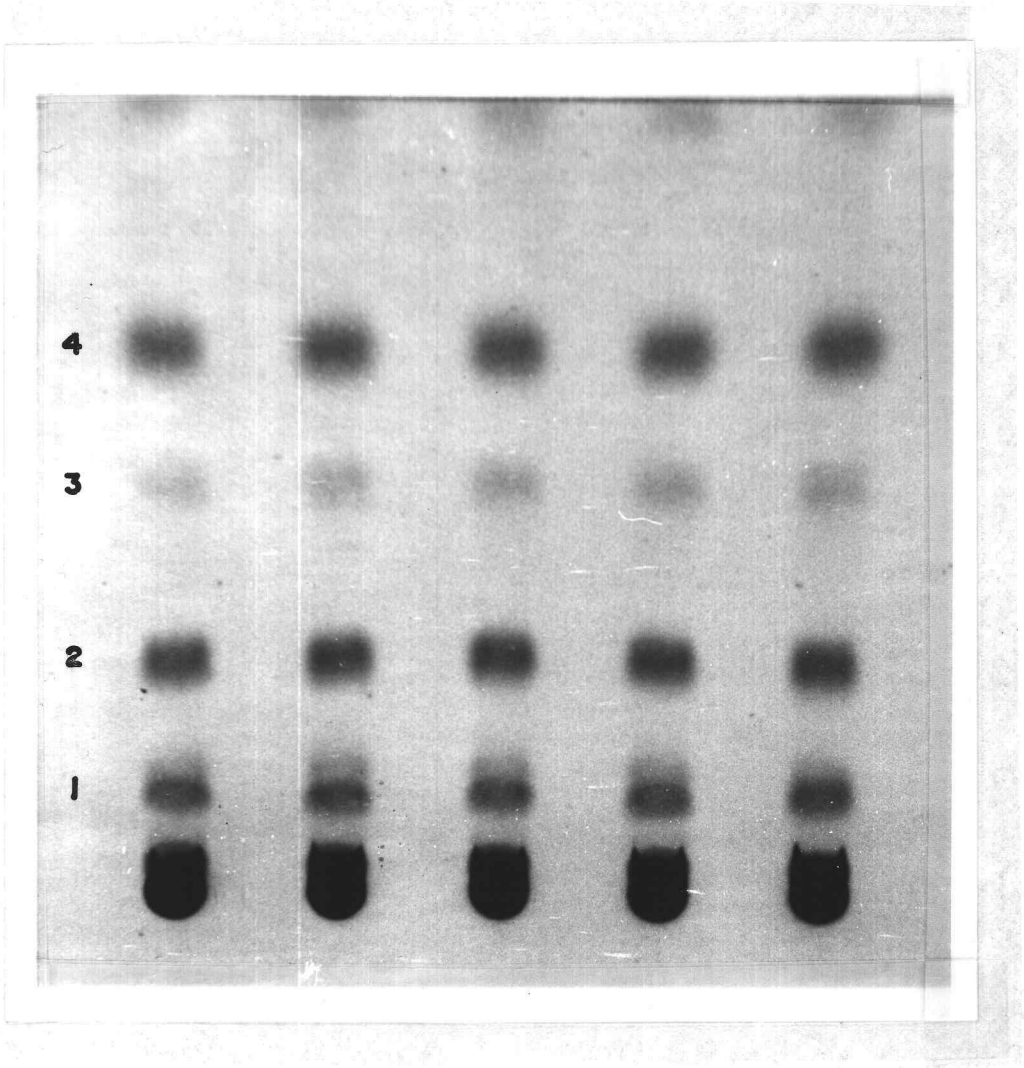

Figure 5. Silica gel thin layer chromatogram of the lipid classes of M. radiodurans. Solvent system: hexane-diethyl ether-acetic acid (88: 12: 1, v/v/v) in a tank development chamber. Visualization: charring with concentrated H_2SO_4 and heating at 200 C for 20 minutes. Doses (left to right): control, 510 krads, 1, 020 krads, 1, 530 krads, and 2, 040 krads.

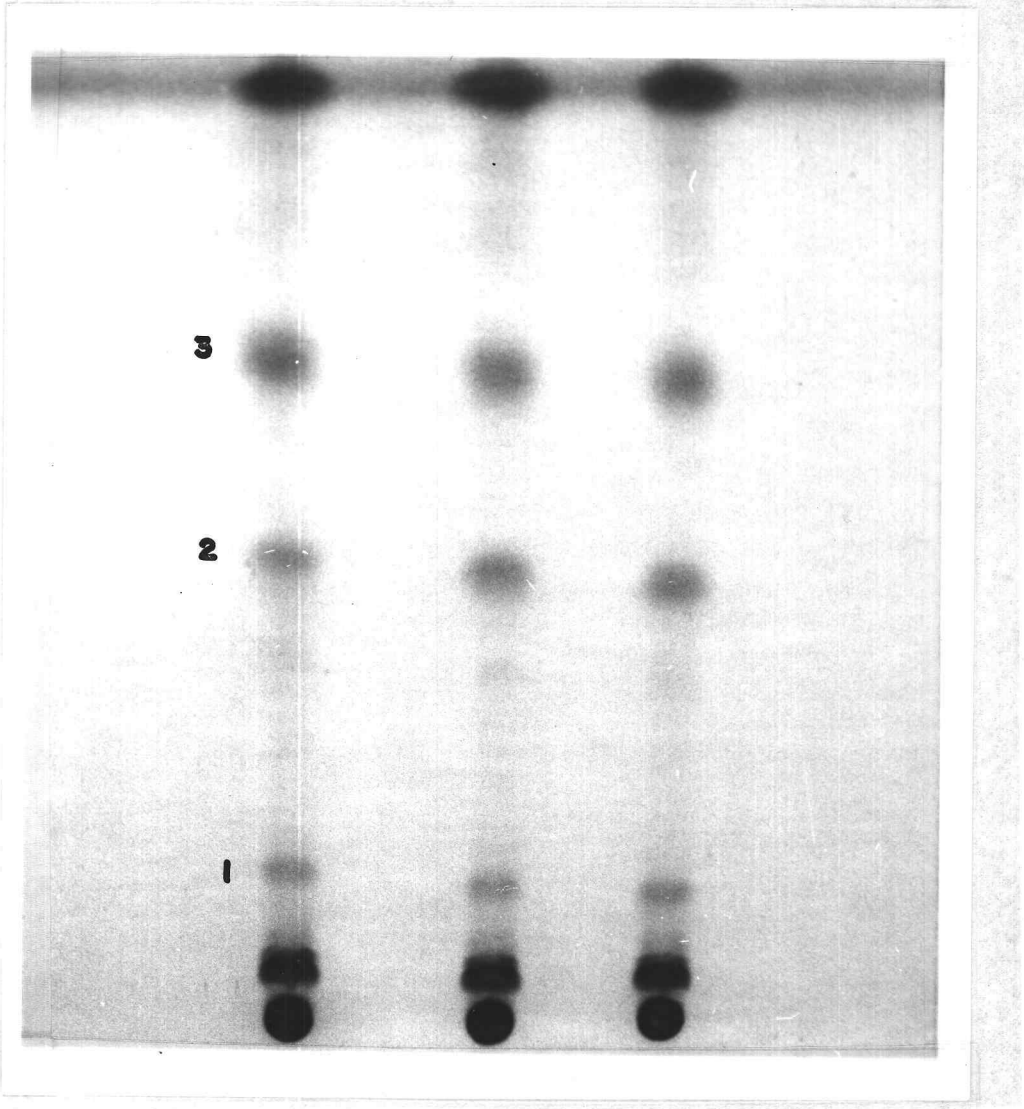

Figure 6. Silica gel thin layer chromatogram of the lipid classes of S. lutea. Solvent system: hexane-diethyl ether-acetic acid (88: 12: 1, v/v/v) in a tank development chamber. Visualization: charring with concentrated H_2SO_4 and heating at 200 C for 20 minutes. Doses (left to right): control, 765 krads, and 2, 040 krads.

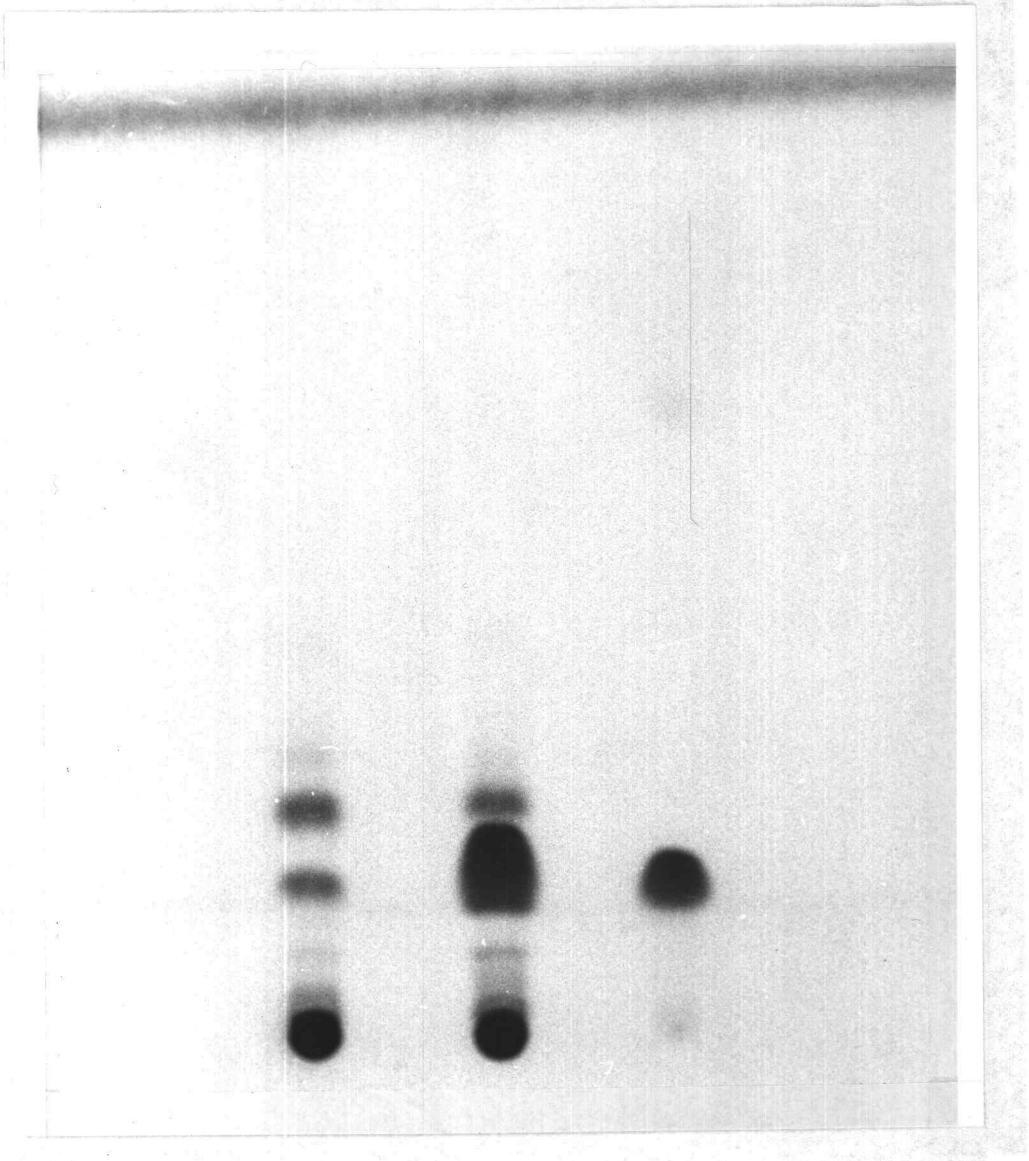

Figure 7. Silica gel thin layer chromatogram of the lipid classes of Ps. aeruginosa. Solvent system: hexane-diethyl ether-acetic acid (88:12:1, v/v/v) in a tank development chamber. Visualization: charring with concentrated H_2SO_4 and heating at 200 C for 20 minutes. (1) control, (2) 2,040 krads, and (3) oleic acid standard. (Left to Right.)

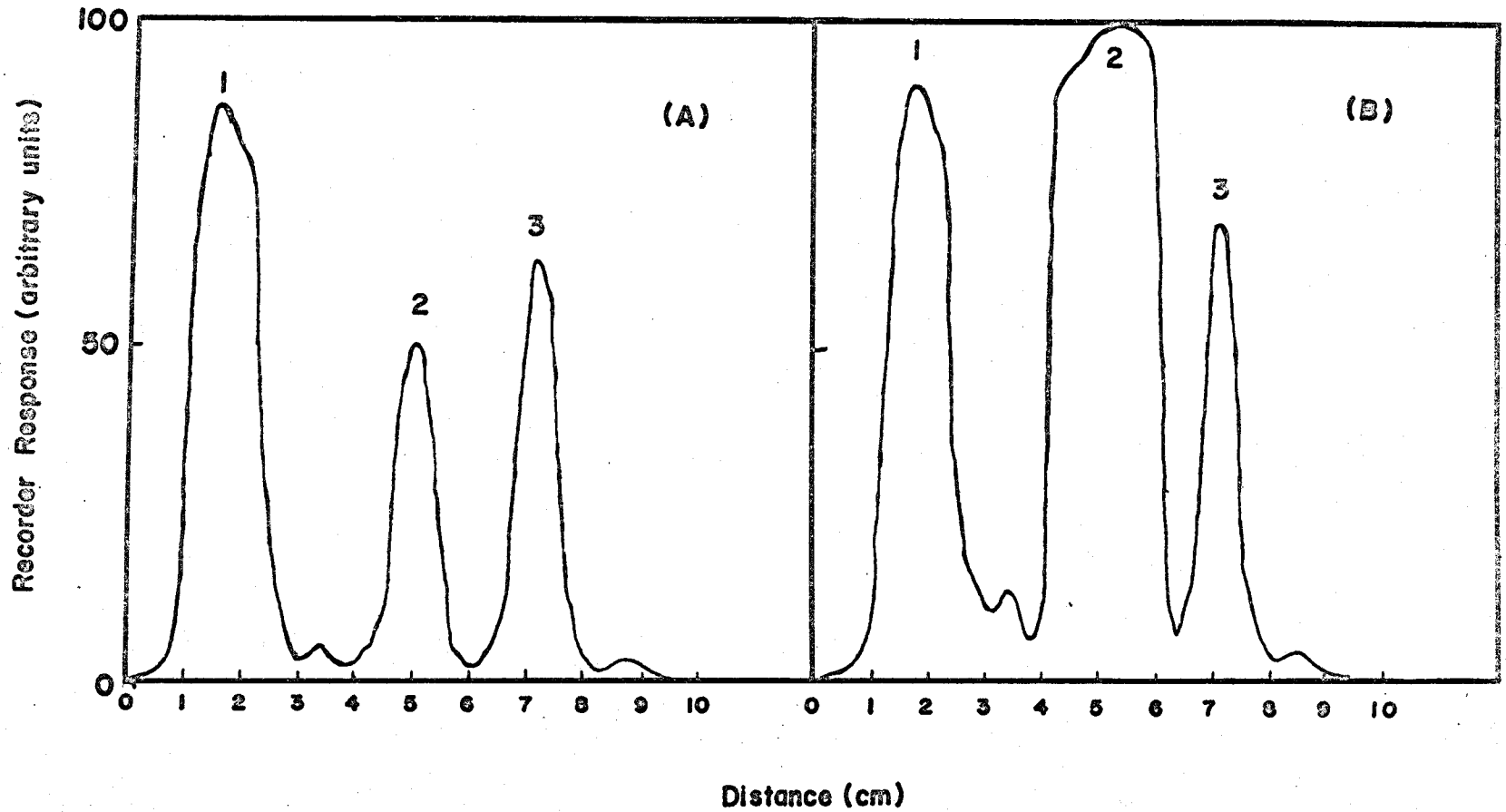

Figure 8. Densitometric recording of a thin layer chromatogram of the lipid classes of Ps. aeruginosa. Peak 2 represents the free fatty acids of (A) unirradiated cells, and (B) cells irradiated at 2, 040 krads.

integrity of cellular membranes during irradiation. The fate of the cell membranes is of importance when considering the enzyme release hypothesis.

Maintenance of membrane integrity could be used to explain the difference in radiation resistance between S. lutea and Ps. aeruginosa, but it does not explain the vast difference in radiation resistance between S. lutea and M. radiodurans. Neither organism displays gross variation in lipid classes following irradiation.

Potassium loss following irradiation has been shown to be far less from M. radiodurans than from S. lutea, indicating that the membrane sensitivities of the cells are different (22). Changes in the membranes of S. lutea which resulted in approximately 75 percent potassium loss from the irradiated cells occurred at a dose less than one-tenth the maximum dose used in this study. Radiation induced changes in the membranes of S. lutea may be more subtle than could be detected in this investigation. The alteration of the saturation status of the fatty acid moieties of the membrane or the formation of stable hydroperoxides could influence membrane permeability following irradiation.

The effect of radiation on the protein portion of the cell membrane should also be considered when investigating permeability changes.

Change in cellular and intracellular permeability is considered

an important primary effect of ionizing radiation (2, p. 272-279). To what extent permeability modifications are responsible for cell death is not known.

This investigation indicates a need for additional research to determine: 1) compounds involved in maintaining cellular membranes; and, 2) the importance of maintaining cellular membranes to cell survival following irradiation.

SUMMARY

Quantity variations in pigments and lipid classes of M. radiodurans, S. lutea, and Ps. aeruginosa following γ-irradiation were determined. The only variation observed in the carotenoid pigmented M. radiodurans and S. lutea was the linear decrease in pigmentation with increased dosage. The neutral lipids and neutral lipid hydrolysates of these two organisms showed no gross variation following a maximum dose of 2,040 krads.

Irradiation of the non-carotenoid pigmented Ps. aeruginosa resulted in a massive increase in free fatty acids upon receiving a maximum dose of 2,040 krads.

A possible role of carotenoid pigments in maintaining membrane integrity is suggested.

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