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LOCALIZATION OF LIPID PEROXIDATION PRODUCTS IN LIPOSOMES AFTER γ-IRRADIATION

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The distribution of lipid peroxidation products in liposomes after γ-irradiation at various doses was studied. Increases in thiobarbituric-acid-reactive substances, in the absorbance at 232 nm and in hydroperoxides were observed mainly in liposomal membranes after relatively low doses of irradiation, while carbonyl compounds were distributed both inside and outside the membranes. After higher doses of irradiation, however, the absorbance at 232 nm and the amount of hydroperoxides reached a maximal level in the membrane portion and then decreased when the decomposition products were released from the membranes. Under this condition, malondialdehyde and other carbonyl compounds were increased mainly in the medium of liposomal suspension. These results are discussed with reference to the lipid peroxidation process which is induced quantitatively by ionizing radiation.

Introduction

There have been many indications of radiation-induced lipid peroxidation in liposomes and biological membranes [1–10]. Previous studies show that radiation causes a permeability increase in liposomes in parallel with the lipid peroxidation, indicating alteration of the interior of the membranes [6–8]. Furthermore, irradiation of the lipid component in microsomes causes a marked decrease in the activity of reconstituted mixed-function oxidase, which indicates a conformational change in membranes upon lipid peroxidation [10]. It has been discovered that the process of lipid peroxidation is initiated by the conjugation of double bonds in polyunsaturated fatty acids of the lipids by radical reactions and is followed by the formation of hydroperoxides and other many kinds of product, including malondialdehyde [11]. However, the localization of each process of lipid peroxidation in the interior or the exterior of membranes has been paid little attention. It is essential to study the distribution of various radiation products in order to elucidate the mechanism of membrane alteration.

The present study deals with quantitative determination of the production and disappearance of conjugated dienes, hydroperoxides, carbonyl compounds, malondialdehyde and their decomposition products after irradiation of liposomes at various doses.

Materials and Methods

Commercial soybean phosphatidylcholine was used after washing three times with acetone as described previously [6]. The acetone-washed phosphatidylcholine was considerably pure by

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thin-layer chromatography. The liposomal suspensions were prepared as described previously [8], except for the general use of 0.15 M KCl/10 mM potassium phosphate buffer (pH 7.4) as the medium.

The liposomal suspensions of 0.5 mg of phosphatidylcholine per ml were irradiated in air at room temperature with about 1500 Ci of ^{60}Co γ-ray beam at either 1.6 Gy/min (lower dose rate, same as in the previous paper [6]) or about 84 Gy/min higher dose rate, which was the maximum rate obtained with the radiation source used. An additional air supply from an air-pump during irradiation up to 2000 Gy produced no substantial effect on the extent of the reaction of the liposomal suspension with thiobarbituric acid.

Radiation-induced lipid peroxidation was determined with various assay methods. The determination of the thiobarbituric acid reaction according to the method of Hunter et al. [12] was performed using the hydrolysate of 1,1,3,3-tetraethoxypropane as a standard. There was no further autoxidation during color development with thiobarbituric acid after irradiation of liposomes, confirmed by the addition of 0.01% butylated hydroxytoluene. Absolute spectra in the ultraviolet region from 300 to 200 nm were determined with a Hitachi 557 double-beam spectrophotometer. The absorption peak at 232 nm appeared to represent conjugated dienes, and the absorption peak at 266 nm represented the formation of malondialdehyde, since the same peak was recognized in the solution of tetraethoxypropane hydrolysate.

The iodometric assay of hydroperoxide was performed according to the method of Buege and Aust [11]. The liposomal suspension, after irradiation, was mixed with 5 vol. chloroform/methanol (2 : 1), followed by centrifugation to separate the phases. After the lower chloroform layer had been recovered and dried, 1 ml of acetic acid/chloroform (3 : 2) and 50 μl of 7.2 M KI were added in the dark, followed by addition of 3 ml of cadmium acetate. The absorbance of the upper phase was determined at 353 nm by use of the molar extinction coefficient of cumene hydroperoxide, $1.73 \cdot 10^{-4}$ M.

Carbonyl compounds were assayed by the formation of 2,4-dinitrophenylhydrazone according to the method of Lappin and Clark [13]. An equal volume of saturated 2,4-dinitrophenylhydrazine in methanol and one drop of concentrated HCl was added to liposomal suspension. After incubation at 50°C for 30 min, 5 ml 10% KOH was added to 2 ml of the incubation mixture and the absorbance of mixture was determined at 480 nm. A mixture containing acetaldehyde in place of liposomal suspensions was used as a standard.

The localization of reaction products in liposomes was determined by two kinds of procedure. First, membranes and surrounding medium were separated by the centrifugation of liposomal suspensions at $105\,000 \times g$ for 1 h after γ-irradiation. Secondly, 1.3 vol. of chloroform/methanol (1 : 0.3) was added to 1 vol. of liposomal suspension and nonpolar and hydrophilic components were extracted into the chloroform and water layers, respectively.

In order to determine the degradation products of malondialdehyde or phosphatidylcholine after irradiation at higher doses, the irradiated tetraethoxypropane hydrolysate and the centrifugal supernatants of the irradiated liposomal suspension were treated with 2,4-dinitrophenylhydrazine, and the dinitrophenylhydrazone thus produced was examined by thin-layer chromatography with chloroform/*n*-hexane (1 : 1) as a solvent system. The spots of these samples were further analyzed by NMR analysis. The NMR spectra were produced by a JEOL FX-270 instrument.

Soybean phosphatidylcholine was purchased from Sigma Chemical Co. 2,4-Dinitrophenylhydrazine was obtained from Daiichi Pure Chemicals Co. Ltd. and 1,1,3,3-tetraethoxypropane from Tokyo Kasei Kogyo Co., Ltd. All other chemicals used in the present study were commercial grade.

Results

Radiation-induced formation and degradation of thiobarbituric acid reactive substances

In order to find the best conditions for the determination of radiation-induced lipid peroxidation, the effect of various medium components in the liposomal suspension on the formation of thiobarbituric acid-reactive substances was examined. Different results for the lipid peroxidation of liposomes were obtained between with the use of 10 mM Tris-HCl buffer and the use of 10 mM

phosphate buffer at pH 7.4 in the presence of 0.15 M KCl or NaCl. Although no substantial difference between either buffer in the lipid peroxidation of liposomes was recognized over the lower range of irradiation dose, drastic increases in the absorbance at 266 nm and in thiobarbituric acid-reactive substances in the presence of Tris-HCl buffer occurred after higher doses of irradiation: about 100 nmol malondialdehyde produced per mg lipid after 2500 Gy irradiation (Fig. 1). The formation of thiobarbituric acid-reactive substances in the presence of phosphate buffer, however, was less evident than that in Tris-HCl buffer; the former reached a maximum level and gradually decreased with increasing radiation dose. Since the production of thiobarbituric acid-reactive substance in Tris buffer was also observed in the absence of liposomes, a large amount of thiobarbituric acid-reactive substance might be produced from Tris itself after the higher dose of irradiation. Therefore, the phosphate buffer medium was employed in the following experiments.

Malondialdehyde prepared as the hydrolysate of tetraethoxypropane decreased after higher doses of irradiation in terms of thiobarbituric acid reactivity and 266 nm absorbance. About 400 μM of malondialdehyde (388.5 $\pm$ 15.0 μM) was decreased to 228.6 $\pm$ 11.7 μM after 1000 Gy irradiation and to 144.2 $\pm$ 9.8 μM after 2000 Gy irradiation. The radiation product from malondialdehyde was examined with the thin-layer chromatography for 2,4-dinitrophenylhydrazone of irradiated malondialdehyde and with the NMR analysis of the sample. The product was determined as acetaldehyde.

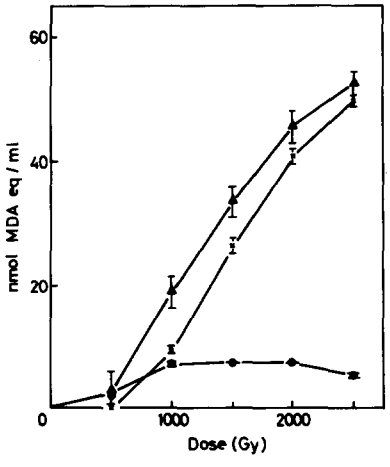

Fig. 1. Radiation-induced changes in thiobarbituric acid-reactive substances from 0.5 mg liposomes per ml 10 mM Tris-HCl (▲) or 10 mM phosphate (●) buffer (pH 7.4) and from 10 mM Tris-HCl buffer (pH 7.4) alone (×). MDA, malondialdehyde. Each point represents an average of three or four experiments ± S.E. (bars).

Lipid peroxidation at a lower irradiation dose

Products of lipid peroxidation from liposomes of soybean phosphatidylcholine after the relatively lower dose of γ-irradiation at 1.6 Gy/min which was used for studies on permeability and lipid peroxidation [6–8] were examined with ultraviolet absorption spectroscopy, thiobarbiaturic acid reaction, iodometric hydroperoxide assay and carbonyl determination. In the ultraviolet absorption spectrum, the absorbance at 232 nm which indicates conjugated dienes increased linearly with increasing doses, while the absorption peak at about 205 nm, which may depend on double bonds, did not alter with the radiaiton dose (Fig. 2A). There was no other peak from 300 to 200 nm after irradiation at a dose lower than 200 Gy, in contrast to the appearance of the absorption peak at 266 nm with the higher doses of irradiation as shown below. Thiobarbituric acid-reactive substances, hydroperoxides and carbonyl compounds in liposomal suspensions were also increased linearly (Figs. 2B, C and D). Control values in non-irradiated liposomal suspensions were 0.252 $\pm$ 0.035 nmol in the case of thiobarbituric acid-reactive substance, 22.2 $\pm$ 2.6 nmol in hydroperoxides and 147 $\pm$ 40 nmol in carbonyl compounds per mg lipid, respectively. The yields of each reaction after 96 Gy of irradiation were remarkably different; thiobarbituric acid-reactive substances were produced to an extent of 1.2 nmol of malondialdehyde equivalent, hydroperoxides were 140 nmol of cumene hydroperoxide equivalent and carbonyl compounds were 340 nmol of acetaldehyde equivalent per mg of lipid, respectively. These results suggest that there may be several kinds of product from lipid peroxidation after γ-irradiation.

Liposomal suspensions irradiated with increasing doses at the lower dose rate were centrifuged at 105 000 × g for 1 h and each product in either supernatants or pellets was determined. The absorbance at 232 nm, thiobarbituric acid-reactive

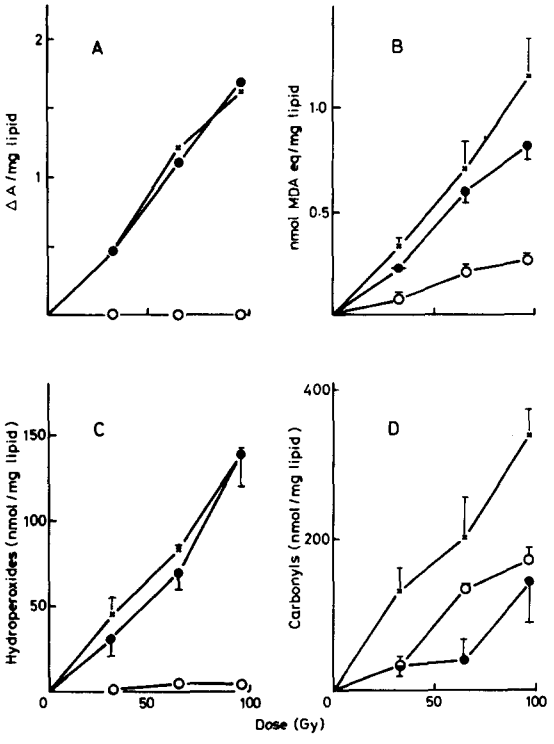

Fig. 2. Lipid peroxidation of liposomal suspensions and in supernatant and pellet fractions after irradiation at the dose rate of 1.6 Gy/min. (A) Radiation-induced changes in the absorbance at 232 nm with total liposomal suspension (×), the pellets after 105 000 × *g* centrifugation (●) and the supernatants (○). The figure shows one experiment from several determinations. (B) Radiation-induced changes in thiobarbituric acid-reactive substances in each fraction. (MDA, malondialdehyde.) (C); Radiation-induced hydroperoxide formation in each fraction of liposomes. (D) Radiation-induced changes in carbonyl compounds in each fraction of liposomes. Each point in (B), (C) and (D) represents an average of three or four experiments ± S.E. (bars).

substances and hydroperoxides were increased in the pellet dose-dependently, but not in the supernatant (Figs. 2A, B and C), though thiobarbituric acid-reactive substances alone appeared slightly in the supernatant. Carbonyl compounds, however, were distributed in both pellet and supernatant (Fig. 2D). From these results, it appears that conjugated dienes and hydroperoxides might be produced in fatty acyl chains of lipids and remain membranes after lower doses of irradiation, but some of the thiobarbituric acid-reactive substances and carbonyl compounds produced in lipid molecules might be excised and transferred to the medium as hydrophilic compounds.

Whether centrifugal fractionation is able to reflect the structural distribution in lipid peroxidation products was examined with the extraction of liposomal suspensions by chloroform/methanol (1 : 0.3) after various doses of irradiation. As shown in Table I, 96 Gy irradiation caused a change in the absorbance at 232 nm accompanied by a slight increase in thiobarbituric acid-reactive substance in the chloroform-soluble fraction, similarly to those in the pellets after the centrifugal fractionation. Since no absorption peak was recognized at 266 nm after 96 Gy irradiation in Table I, absorption values at 266 nm in the lower dose of irradiation might not indicate the formation of malondialdehyde.

Lipid peroxidation at a higher irradiation dose

Lipid peroxidation in liposomes irradiated at about 84 Gy/min was assayed under the same conditions as in the case of the lower dose of irradiation. The absorbance at 232 nm was increased rapidly until 500 Gy, but it then altered to be decreased with further increasing doses (Fig. 3A). On the other hand, the peak of absorbance at 266 nm appeared and increased with more than 500 Gy, but it reached a maximum at 1500–2000 Gy.

Radiation-induced production of hydroperoxides reached a maximum at 500 Gy and decreased with further increasing doses, as did the absorbance at 232 nm (Figs. 3A and C). The increase in thiobarbituric acid-reactive substances until 1000 Gy irradiation was in parallel with the increase in the absorbance at 266 nm (Fig. 3A and B). Carbonyl compounds, however, were sharply increased upto 500 Gy and gradually increased further with increasing doses (Fig. 3D).

When liposomes were fractionated by centrifugation into pellets and supernatants after irradiation at doses higher than 500 Gy, the absorbance at 266 nm in the supernatant increased gradually with more than 500 Gy and the absorbance at 232 nm in the pellet reached a maximum at 500 Gy (Fig. 3A). Although the production of thiobarbituric acid-reactive substances in pellets was a little more than that in supernatants after irradiation at less than 500 Gy, the further increase in thiobarbituric acid-reactive substance was observed only in

TABLE I

LIPID PEROXIDATION OF LIPOSOMAL SUSPENSIONS AND THEIR CHLOROFORM EXTRACTS AFTER IRRADIATION

Liposomal suspensions of 0.5 mg phosphatidylcholine per ml of 0.15 M KCl/10 mM phosphate buffer (pH 7.4) were extracted with the additions of 1 part of chloroform and 0.3 part of methanol after 96 and 1000 Gy irradiation. Radiation-induced formation in thiobarbituric acid-reactive substances and radiation-induced changes in the absorbance at 232 nm and 266 nm were measured in total suspension and in water-soluble and chloroform-soluble fractions. Each value represents the average of three experiments ± S.E.

Measurements	Dose (Gy)	Total liposomes	Water-soluble fraction	$CHCl_3$-soluble fraction
			nmol/mg lipid	
Thiobarbituric acid reaction	96	1.25 ± 0.13	0.08 ± 0.00	0.78 ± 0.07
	1000	1.40 ± 0.17	8.61 ± 0.15	0.81 ± 0.03
			ΔA/mg lipid	
ΔA_{232}	96	3.24 ± 0.36	0.00 ± 0.00	2.45 ± 0.35
	1000	4.31 ± 0.00	0.11 ± 0.04	2.61 ± 0.21
ΔA_{266}	96	0.22 ± 0.01	0.00 ± 0.00	0.14 ± 0.09
	1000	0.91 ± 0.02	0.40 ± 0.02	0.17 ± 0.06

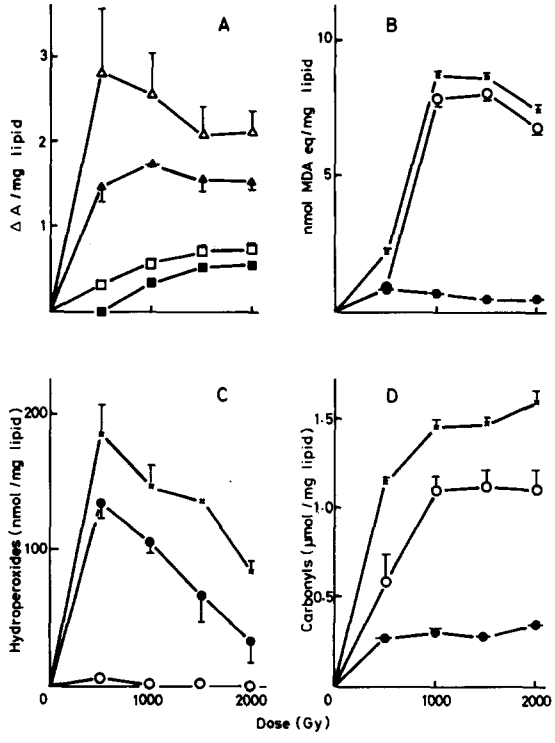

Fig. 3. Lipid peroxidation in supernatant and pellet fractions of liposomal suspensions after irradiation at a dose rate of about 84 Gy/min. (A) Radiation-induced changes in the absorbance at 232 nm in total liposomes (△) and pellets (▲) and in the absorbance at 266 nm in total liposomes (□) and supernatants (■). (B) Radiation-induced changes in thiobarbituric acid-reactive substances in total liposomes (×), pellets after 105000 × g centrifugation (●) and the supernatants (○) (MDA, malondialdehyde). (C) Radiation-induced changes in hydroperoxides in each fraction. (D) Radiation-induced changes in carbonyl compounds in each fraction. Each point represents an average of three or four experiments ± S.E. (bars).

supernatants after more than 500 Gy (Fig. 3B). These results indicate that most of thiobarbituric acid-reactive products after higher doses of irradiation might be released from membrane lipids and transferred into the medium as soluble molecules. Indeed, malondialdehyde prepared from the hydrolysis of 1,1,3,3-tetraethoxypropane had a specific peak at 266 nm and the extent was dependent on the concentration. The soluble thiobarbituric acid-reactive product, therefore, was recognized as malondialdehyde. Hydroperoxides produced after higher doses of irradiation localized absolutely in the pellet, as did the absorbance at 232 nm (Fig. 3C). On the other hand, carbonyl compounds were increased considerably in the supernatant after higher doses of irradiation in comparison with the pellet (Fig. 3D).

The extraction from liposomal suspension by chloroform/methanol (1 : 0.3) after 1000 Gy of irradiation is shown in Table I. Although the increase in thiobarbituric acid-reactive substance in total liposomal suspensions was accompanied with increases in the absorbances at both 232 nm and at 266 nm, it is evident that at higher doses of

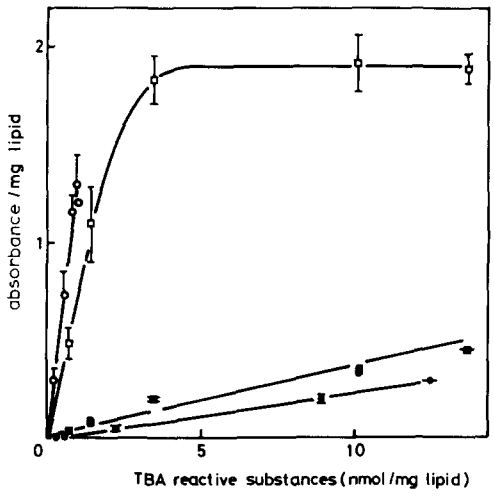

Fig. 4. Correlation between the formation of thiobarbituric acid (TBA)-reactive substances and changes in ultraviolet absorbance after various doses of irradiation. The absorbance at 232 nm is represented in total liposomal suspensions (□) and in centrifugal pellets (○). The absorbance at 266 nm is represented in total suspensions (■) and in centrifugal supernatants (●).

irradiation the radiation products consisted mainly of the water-soluble component, malondialdehyde.

As shown in Fig. 4, two kinds of thiobarbituric acid-reactive substance produced after irradiation were also indicated from the correlation between thiobarbituric acid reactivity and two peaks of ultraviolet absorption with regard to radiation dose and localization. It was shown clearly that the increase in thiobarbituric acid-reactive substances correlated with that in the absorbance at 232 nm, conjugated dienes, in liposomal membranes in the lower range of thiobarbituric acid reactivity. In the higher dose range, however, the absorbance at 232 nm reached a plateau level against the increase in thiobarbituric acid-reactive substances, in contrast to the continuous increase in the absorbance at 266 nm.

Discussion

Radiation-induced lipid peroxidation is thought to induce the cellular deterioration through membrane damage. Since the extent of lipid peroxidation has been shown to be dependent on the radiation dose [2–8], the mechanism of membrane alteration can be analysed by the estimation of the chemical process of lipid peroxidation using the quantitative feature of ionizing radiation. The radiation-induced lipid peroxidation has been determined mainly by two methods, namely specific ultraviolet absorption at 232 nm [1,3,4,14,15] and the thiobarbituric acid reaction [2,5,6]. Although these values could reflect most of the radiation-induced damage in lipid bilayers [1–8,10,11], the changes in various parameters of lipid peroxidation must be compared with each other, because there are many kinds of decomposition products in lipid peroxidation [16]. From the present results the interrelationship among products of lipid peroxidation, thiobarbituric acid-reactive and ultraviolet-absorbing substances, hydroperoxides and carbonyl compounds, is indicated with reference to the radiation sensitivity and the localization of products.

The radiation-induced chemical process of membrane damages might be estimated by the analysis of the localization of lipid peroxidation products. The present results indicate the production of a thiobarbituric acid-reactive substance in liposomal membranes by irradiation in the lower dose range without the formation of malondialdehyde, the release of malondialdehyde from lipid bilyaers through the formation of conjugated dienes or hydroperoxides by the higher doses and, furthermore, degradation of malondialdehyde to produce acetaldehyde. According to Kunimoto et al. [17] thiobarbituric acid-reactive substances formed in liposomes by the treatment of ferrous ion and ascorbate were released from membranes. This result indicates in reference to the present study that the released substances may be malondialdehyde or other carbonyl compounds, though these latter compounds are reported not to react with thiobarbituric acid [18]. It has been shown that the peroxidation of unsaturated fatty acids and microsomal lipids leads to the production of a great diversity of polar or nonpolar carbonyls [18]. From the present results, the molecular yield of carbonyl compounds is highest among lipid peroxidation products tested and the distribution of carbonyl compounds in both pellets and supernatants of irradiated liposomes suggests the production of polar and nonpolar aldehydes in liposomes. These results indicate that the production of carbonyl compounds should be an essential

step of radiation-induced lipid peroxidation as well as the formation of hydroperoxides.

Radiation-induced lipid peroxidation is accompanied with changes in physiological features of membranes. The permeability of ions and non-electrolytes is increased linearly with increasing radiation dose of ionizing [6,19,20] and ultraviolet [14] irradiation. The irradiation of liposomes below 100 Gy causes a linear increase in both glucose permeability and production of carbonyl or hydroperoxide groups which might induce the hydrophobic region of membranes to change the hydrophilic environment and to increase the permeability of glucose. The permeability increase is also observed in the lipid peroxidation induced by lipoxygenase [21]. Radiation-induced changes in the physical state of liposomal membranes will be presented in near future.

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References

1 Mead, J.F. (1952) Science 115, 470–472
2 Wills, E.D. (1970) Int. J. Radiat. Biol. 17, 217–228
3 Petkau, A. and Chelack, W.S. (1976) Biochim. Biophys. Acta 443, 445–456
4 Raleigh, J.A., Kremers, W. and Gaboury, B. (1977) Int. J. Radiat. Biol. 32, 203–213
5 Yukawa, O. and Nakazawa, T. (1980) Int. J. Radiat. Biol. 37, 621–631
6 Nakazawa, T. and Nagatsuka, S. (1980) Int. J. Radiat. Biol. 38, 537–544
7 Nakazawa, T., Nagatsuka, S. and Sakurai, T. (1981) Int. J. Radiat. Biol. 40, 365–373
8 Nagatsuka, S. and Nakazawa, T. (1982) Biochim. Biophys. Acta 691, 171–177
9 Nakazawa, T., Yukawa, O., Nagatsuka, S., Matsudaira, H. and Sato, K. (1982) Int. J. Radiat. Biol. 42, 581–586
10 Yukawa, O. and Nakazawa, T. (1983) Int. J. Radiat. Biol. 43, 391–398
11 Buege, J.A. and Aust, S.O. (1978) Methods Enzymol. 52, 302–310
12 Hunter, F.E., Jr., Gebicki, J.M., Hoffsten, P.E., Weinstein, J. and Scott, A. (1963) J. Biol. Chem. 238, 828–835
13 Lappin, G.R. and Clark, L.C. (1951) Anal. Chem. 23, 541–542
14 Mandal, T.K., Ghose, S., Sur, P. and Chatterjee, S.N. (1978) Int. J. Radiat. Biol. 33, 75–79
15 Konings, A.W.T., Damen, J. and Trieling, W.B. (1979) Int. J. Radiat. Biol. 35, 343–350
16 Gardner, H.W. (1975) J. Agric. Food Chem. 23, 129–136
17 Kunimoto, M., Inoue, K. and Nojima, S. (1981) Biochim. Biophys. Acta 646, 169–178
18 Esterbauer, H., Cheeseman, K.H., Dianzani, M.U., Poli, G. and Slater, T.F. (1982) Biochem. J. 208, 129–140
19 Myers, D.K. and Bide, R.W. (1966) Radiat. Res. 27, 250–263
20 Konings, A.W.T. (1981) Int. J. Radiat. Biol. 40, 441–444
21 Hicks, M. and Gebicki, J.M. (1978) Biochem. Biophys. Res. Commun. 80, 704–708