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EVALUATION OF ADRIAMYCIN-INDUCED LIPID PEROXIDATION

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Abstract—Lipid peroxidation is known to be a mechanism for Adriamycin®-induced toxicity. In the present study, two methods which detect fluorescent substances and high molecular weight protein aggregates in peroxidized membranes were applied to Adriamycin-induced lipid peroxidation in liver microsomes. A rat liver microsomal suspension containing an NADPH-generating system was incubated with Adriamycin. Thiobarbituric acid reactive substances (TBA-RS), formed during this incubation, were transferred from the microsomes to the medium. Fluorescent substances determined by the fluorescence emitted from both the microsomes themselves and the chloroform/methanol extracts of the microsomes, were found to be formed during this incubation. High molecular weight protein aggregates determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, were also formed. Fluorescent substances and high molecular weight protein aggregates were found in microsomal membranes themselves and increased time dependently. These substances retained in membranes can be of great use to delineate the site of Adriamycin-induced lipid peroxidation *in vitro* and *in vivo* and to determine how this lipid peroxidation affects the membrane.

Adriamycin® is an antineoplastic agent which is widely used in the treatment of a variety of human cancers. Together with valid therapeutic activity, this drug is well known to have adverse effects. Adriamycin is activated to the semiquinone free radical by microsomal NADPH–cytochrome P450 reductase [1, 2] and induces NADPH-dependent lipid peroxidation in microsomes mediated by reactive oxygen [3, 4].

Adriamycin-induced lipid peroxidation has been evaluated by thiobarbituric acid reactive substances (TBA-RS) and alkanes such as ethane and pentane. The former is generally used as an indicator of *in vitro* and *in vivo* lipid peroxidation. The latter is a useful *in vivo* indicator [5] and is also used as an *in vitro* indicator [6]. Although these indicators are very helpful in detecting the occurrence of lipid peroxidation, they may not precisely delineate the *in vivo* site where lipid peroxidation occurs, because both TBA-RS and alkanes leave their sites of formation and appear in the blood stream, in the urine and in expired air. In our earlier report, we suggested this possibility by characterizing the formation of TBA-RS in NADPH/Fe^{2+}-induced lipid peroxidation with rat liver microsomes [7].

The formation of fluorescent substances during lipid peroxidation has been observed in artificial phospholipid membranes [8] and biological membranes such as liver microsomes [9] and hepatocytes [10]. The fluorescent substances are retained in the membranes [7], and may be a useful indicator for the peroxidation reaction site. In these studies, lipid peroxidation was induced with ascorbic acid/Fe^{2+}, ADP/Fe^{3+} or cumene hydroperoxide. High molecular weight protein aggregates formed during lipid peroxidation are also retained in the membranes [9, 10]. However, the fluorescent substances and the high molecular weight protein aggregates formed during lipid peroxidation induced by clinically used drugs have not been studied extensively thus far. Consequently, additional information on drug-induced lipid peroxidation would be helpful for our understanding of drug-induced toxicity.

To clarify further the contribution of lipid peroxidation to Adriamycin-induced toxicity *in vivo* and *in vitro*, it is essential to determine the specific site where lipid peroxidation occurs and to evaluate the peroxidation quantitatively and qualitatively. Thus, this study was intended to establish a method to substantially prove the site of Adriamycin-induced lipid peroxidation and to evaluate the change in membranes induced by lipid peroxidation.

MATERIALS AND METHODS

Materials. NADP was purchased from the Oriental Yeast Co. Ltd. (Tokyo, Japan). Glucose-6-phosphate disodium salt and glucose-6-phosphate dehydrogenase (type VII) were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Heparin sodium salt was from the Novo Industries Co. (Denmark). An electrophoresis calibration kit (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactoalbumin) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Adriamycin hydrochloride was supplied by the Kyowa Hakko Kogyo Co. (Tokyo, Japan). All other reagents were of the highest purity available.

Preparation of liver microsomes. Male Wistar rats (Japan SLC Inc., Shizuoka, Japan), weighing 200–250 g, were kept in the laboratory for at least 2 weeks before they were used to prepare the liver

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microsomes. The rats were anesthetized with ethyl ether and given an injection of heparin (200 U in 0.2 mL). The livers were then perfused through the portal vein with ice-cold saline solution and excised. The isolated livers were minced with scissors and homogenized in an isotonic KCl solution, using a teflon homogenizer. The homogenate was diluted 1 to 7 and then centrifuged at 60 *g* for 7 min at 4°. The supernatant was centrifuged at 600 *g* for 7 min at 4°. The supernatant was further centrifuged at 7,700 *g* for 10 min at 4°. Finally, the supernatant was centrifuged at 105,000 *g* for 1 hr at 4°. The microsomal pellet was resuspended in 0.15 M KCl, 0.05 M Tris–HCl buffer, pH 7.4. The protein concentration of microsomes was determined by the method of Lowry *et al.* [11] using bovine serum albumin as the standard.

Reaction of microsomes with Adriamycin. The reaction of microsomes with Adriamycin was carried out according to a slight modification of the method of Mimnaugh *et al.* [4]. The KCl (0.15 M)–Tris (0.05 M)–HCl buffer, pH 7.4, was oxygenated by bubbling the gases (95% O_2–5% CO_2). Liver microsomes were suspended in this oxygenated buffer and incubated with Adriamycin for 5 min at 37°. Then the reaction was started at 37° by adding an NADPH-generating system consisting of 1.9 mM NADP, 20 mM glucose-6-phosphate, 1.1 U/mL glucose-6-phosphate dehydrogenase, and 8.6 mM $MgCl_2$ to the microsomal suspension. The medium was gassed with 95% O_2–5% CO_2, and then the flasks containing the reaction mixture were covered with plastic film. The reactions were terminated by adding 1 mM EDTA at the designated times. The reaction mixtures were protected against light exposure throughout.

Lipid peroxidation. Lipid peroxidation of microsomes (0.2 mg protein/mL) was induced by incubating the microsomal suspension with 0.2 mM NADPH and 20 μM $FeSO_4$ or 0.1 mM ascorbic acid and 5 μM $FeSO_4$ for 1 hr at 37° and terminated by the addition of 1 mM EDTA as reported elsewhere [7].

Assay for TBA-RS. TBA-RS formed in the reaction mixture was assayed according to the method of Buege and Aust [12] and expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of protein.

Assay for fluorescent substances. The reaction mixtures were centrifuged at 105,000 *g* for 60 min at 4° and the supernatant was discarded to remove NADPH. The pellet was resuspended in the buffer. The fluorescent substances were detected in two ways as reported elsewhere [7]: (i) the fluorescence was measured directly in peroxidized microsomes, using excitation and emission wavelengths of 370 and 440 nm, respectively, and (ii) the fluorescence was measured in chloroform/methanol extracts of microsomes according to the procedures of Koster and Slee [9] and Fletcher *et al.* [13], using excitation and emission wavelengths of 361 and 437 nm, respectively. The samples in cuvettes, in either case, were irradiated with ultraviolet light to remove the fluorescence contribution in microsomes of such compounds as retinol, using a sterilizing lamp (Toshiba GL 10; wavelength, 253.7 nm; output, 1.8 W) just before fluorescence measurements. Fluorescence measurements were carried out at 25°, using a Hitachi fluorescence spectrophotometer 650–60. A cutoff filter (390 nm) was placed on the emission side for each measurement.

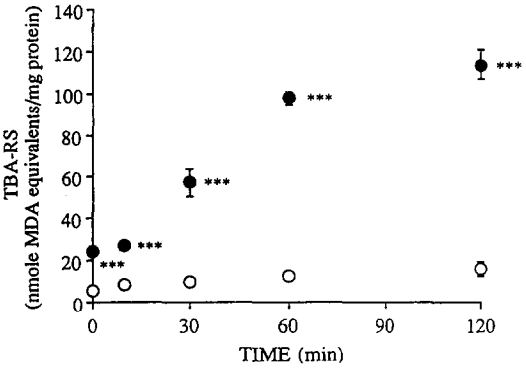

Fig. 1. Effect of incubation time on Adriamycin-induced lipid peroxidation in liver microsomes. Liver microsomes (0.2 mg protein/mL) were incubated with an NADPH-generating system in the presence (●) and absence (○) of 100 μM Adriamycin. Lipid peroxidation was evaluated by TBA-RS formation. The data points represent the means ± SEM of three experiments. Where absent, SEMs were smaller than the symbols. A triple asterisk (***) indicates a value significantly different from the data without Adriamycin ($P < 0.001$).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Weber and Osborn [14] as described elsewhere [15]. The samples were applied to the gels after being mixed with 0.2 M phosphate buffer (pH 7.4) containing 5% SDS, 20% glycerol, 2 mM 2-mercaptoethanol and 0.06% phenol red. The gel bands stained with 0.1% Coomassie Brilliant Blue were detected using a Shimazu chromatoscanner CS-9000 by double wavelengths (550 nm as a sample and 490 nm as a reference).

Statistical analysis. Statistical analysis was performed by Student's *t*-test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Formation of TBA-RS. Experimental conditions necessary for the study of *in vitro* Adriamycin-induced lipid peroxidation were determined in liver microsomes by measuring TBA-RS formed in the reaction mixture. TBA-RS formation was dependent on incubation time and microsomal concentration (Figs. 1 and 2). TBA-RS formation increased with increasing Adriamycin concentration and reached a plateau above 200 μM (Fig. 3). Based on these results, the present experiments were carried out at 0.2 mg protein/mL of microsomes, 100 μM Adriamycin and an incubation time of 1 hr.

Formation of fluorescent substances. NADPH was removed by centrifugation from the microsomal

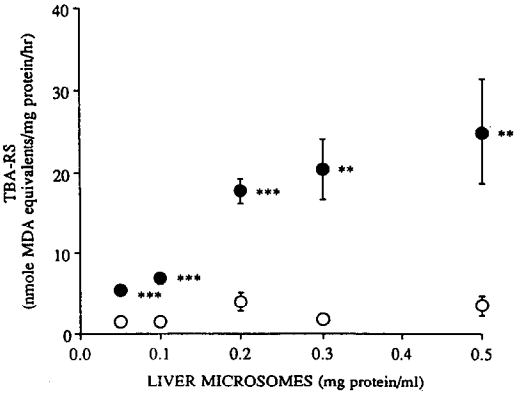

Fig. 2. Effect of liver microsomal concentration on Adriamycin-induced lipid peroxidation. Liver microsomes were incubated with 100 μM Adriamycin and an NADPH-generating system. Incubation time: 0 hr (○); 1 hr (●). The data points represent the means ± SEM of six experiments. Where absent, SEMs were smaller than the symbols. Values marked by a double or triple asterisk were significantly different from the data incubated for 0 hr: (**) $P < 0.01$, and (***) $P < 0.001$.

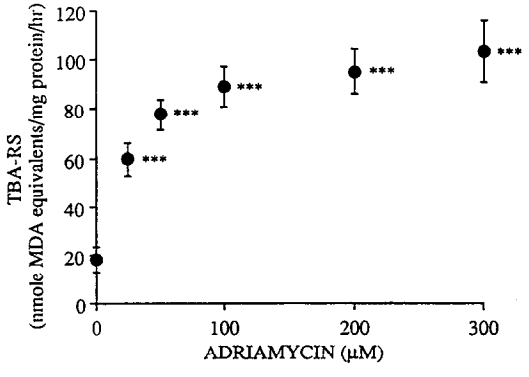

Fig. 3. Effect of Adriamycin concentration of Adriamycin-induced lipid peroxidation in liver microsomes. Liver microsomes (0.2 mg protein/mL) were incubated for 1 hr with an NADPH-generating system. Lipid peroxidation was evaluated by TBA-RS formation. The data show the amount of TBA-RS in the reaction mixture at 1 hr minus that at zero time for each point. The data points represent the means ± SEM of six experiments. Where absent, SEMs were smaller than the symbols. A triple asterisk (***) indicates a value significantly different from the data without Adriamycin ($P < 0.001$).

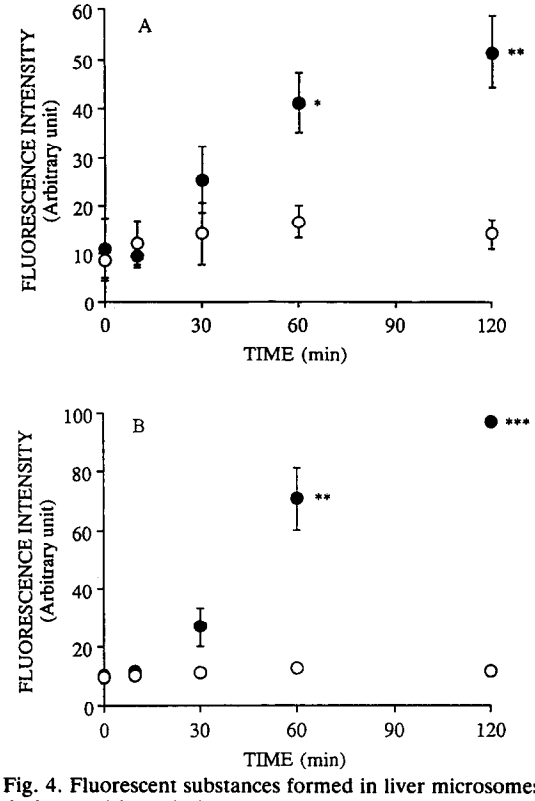

Fig. 4. Fluorescent substances formed in liver microsomes during Adriamycin-induced lipid peroxidation. Liver microsomes (0.2 mg protein/mL) were incubated for 1 hr with an NADPH-generating system in the presence (●) and absence (○) of 100 μM Adriamycin. Formation of fluorescent substances was demonstrated by the fluorescence intensity. (A) Fluorescence emitted from the microsomes. (B) Fluorescence emitted from the chloroform/ethanol extracts of the microsomes. The data points represent the means ± SEM of three experiments. Where absent, SEMs were smaller than the symbols. Values marked by a single, double or triple asterisk were significantly different from the data without Adriamycin: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$.

suspension which had been incubated with Adriamycin, because the fluorescence measurements were disturbed by the NADPH fluorescence. Fluorescent substances in the separated microsomes were examined by measuring the fluorescence emitted from the microsomes themselves and from chloroform/methanol extracts of the microsomes. The fluorescence from the microsomes themselves showed a continuous increase with incubation time (Fig. 4A), as did the fluorescence from the extracts (Fig. 4B).

Formation of high molecular weight protein aggregates. The molecular distribution of proteins in peroxidized microsomes incubated with Adriamycin was examined using SDS–PAGE. The electrophoretic patterns of SDS–PAGE determined by a chromatoscanner are shown in Fig. 5. The high molecular weight proteins at the top of the gel rod, in the microsomes incubated for 1 hr, increased remarkably, while the proteins around the middle of the gel rod decreased. The formation of high molecular weight protein aggregates in microsomes in the presence of Adriamycin proceeded with incubation time (Fig. 6).

Distribution of TBA-RS and high molecular weight protein aggregates in the microsomal suspension. The distribution in the microsomal suspension of TBA-RS and high molecular weight protein aggregates

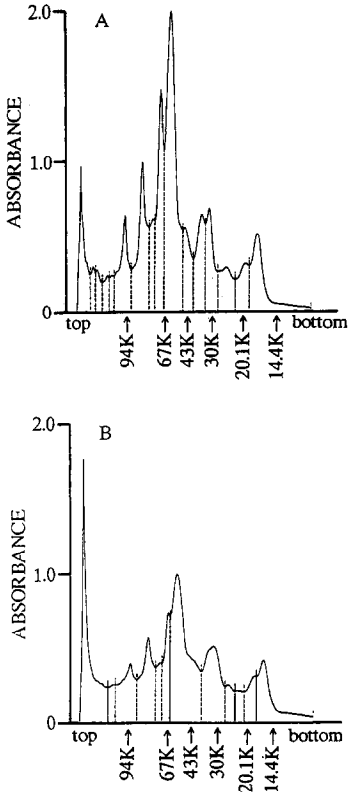

Fig. 5. SDS–PAGE of liver microsomes. Liver microsomes (0.2 mg protein/mL) were incubated for 1 hr with 100 μM Adriamycin and an NADPH-generating system. The microsomal proteins were separated by SDS–PAGE and the gels were stained with Coomassie Brilliant Blue. The stain intensity in the gels was measured by a chromatoscanner and the absorbance is expressed on the ordinate of the figure. (A) Microsomes at zero time in the presence of 100 μM Adriamycin. (B) Microsomes incubated for 1 hr in the presence of 100 μM Adriamycin. Standard proteins: phosphorylase *b* (M_r 94 K); bovine serum albumin (M_r 67 K); ovalbumin (M_r 43 K); carbonic anhydrase (M_r 30 K); soybean trypsin inhibitor (M_r 20.1 K); and α-lactoalbumin (M_r 14.4 K).

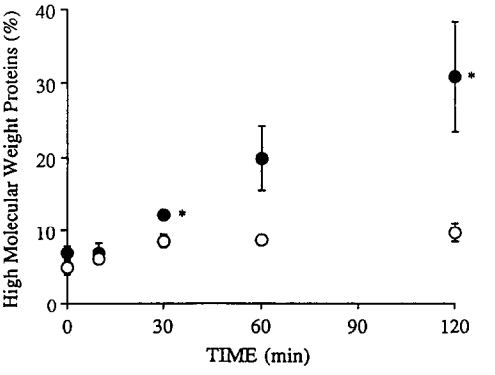

Fig. 6. High molecular weight protein aggregates formed in liver microsomes during Adriamycin-induced lipid peroxidation. Liver microsomes (0.2 mg protein/mL) were incubated with an NADPH-generating system in the presence (●) and absence (○) of 100 μM Adriamycin. The gel bands of separated microsomal proteins were stained with Coomassie Brilliant Blue. High molecular weight proteins which appeared at the top of the gels were expressed as a percentage (%) of the amount of total proteins. The data points represent the means ± SEM of three experiments. Where absent, SEMs were smaller than the symbols. An asterisk (*) indicates a value significantly different from the data without Adriamycin ($P < 0.05$).

formed during Adriamycin-induced lipid peroxidation was investigated. The reaction mixtures at each time point were separated into the pellets and the supernatants by centrifuging the mixtures at 105,000 *g* for 1 hr. TBA-RS determination and SDS–PAGE were carried out on the supernatants and the pellets. The greater part of TBA-RS appeared in the supernatants, whereas the amount of TBA-RS in the pellets was very small (Fig. 7A). A greater part of high molecular weight protein aggregates appeared in the pellets, but little increase in the high molecular weight protein aggregates was observed in the supernatants (Fig. 7B).

Lipid peroxidation induced by Adriamycin and other peroxidation systems. Adriamycin-induced lipid peroxidation was compared with enzymatically (NADPH and Fe^{2+})- and non-enzymatically (ascorbic acid and Fe^{2+})-induced lipid peroxidation (Table 1). The microsomes (0.2 mg protein/mL) in each peroxidation system were incubated for 1 hr at 37°. It should be noted that Adriamycin showed a markedly increased production of TBA-RS, fluorescent substances and high molecular weight proteins, compared to those of the control. Further, the increase was more remarkable than those produced by other typical peroxidation systems. This indicated that the fluorescent substances and high molecular weight protein aggregates were reliable indicators in evaluating the Adriamycin-induced lipid peroxidation.

DISCUSSION

This work was intended to develop a method to characterize Adriamycin-induced lipid peroxidation. Optimal experimental conditions to study Adriamycin-induced lipid peroxidation were determined by monitoring TBA-RS, a generally used indicator of lipid peroxidation (Figs. 1–3).

We recently developed a new method to detect fluorescent substances formed in peroxidized membranes, using rat liver microsomes [7]. This technique was applied to Adriamycin-induced lipid peroxidation in liver microsomes (Fig. 4). Since the fluorescence of NADPH contained in the reaction mixtures disturbed the fluorescence measurement, the pellets separated by centrifuging the reaction mixtures were used for this study. The fluorescence emitted from the microsomes increased with the incubation time (Fig. 4A). The organic solvent-extracts also showed a similar time-dependent increase in fluorescence (Fig. 4B). The fluorescent substances in the reaction mixtures and in their centrifuged supernatants were not determined,

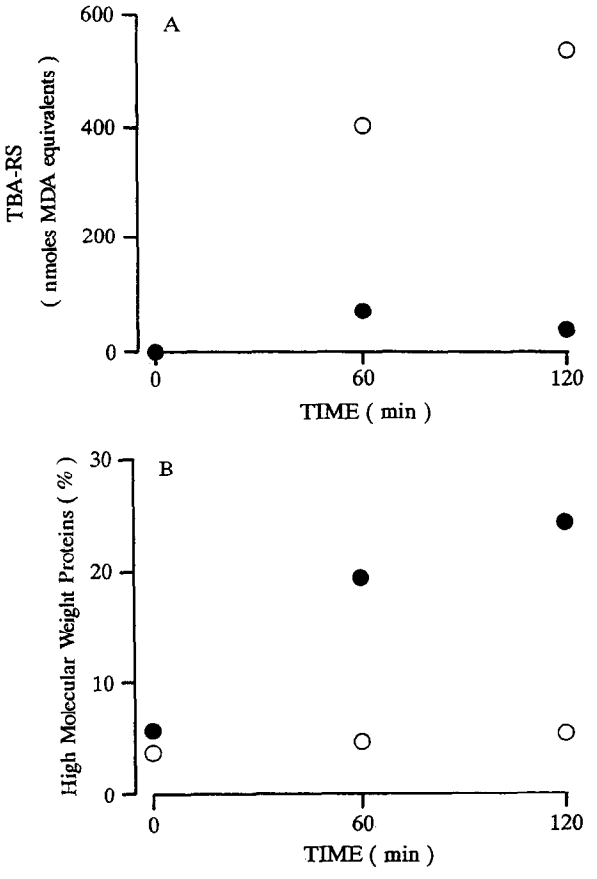

Fig. 7. Distribution of TBA-RS and high molecular weight proteins in the microsomal suspension during Adriamycin-induced lipid peroxidation. Liver microsomes (0.2 mg protein/mL) were incubated for 0, 1 and 2 hr with 100 μM Adriamycin and an NADPH-generating system. The reaction mixture was, after termination of the reaction, separated into the pellets (microsomes) and the supernatants by centrifuging the mixture at 105,000 g for 1 hr. The pellets were resuspended in the buffer. Key: (A) TBA-RS, and (B) high molecular weight protein aggregates in the resuspended pellets (●) and in the supernatants (○).

Table 1. Formation of TBA-RS, fluorescent substances and high molecular weight protein aggregates in liver microsomes

Incubation system	TBA-RS (nmol MDA equivalents/mg protein)	Fluorescent substances (A) (B) Fluorescence intensity (arbitrary unit)		High molecular weight proteins (%)
Control (microsomes alone)	12.0 ± 1.0 (3)	16.4 ± 3.4 (3)	12.6 ± 1.1 (3)	8.0 ± 0.6 (5)
+ Adriamycin (100 μM) with the NADPH-generating system	97.4 ± 3.1* (3)	41.1 ± 6.1† (3)	70.5 ± 10.5‡ (3)	27.7 ± 2.9* (8)
+ NADPH (0.2 mM) and Fe^{2+} (20 μM)	63.8 ± 1.0* (3)	23.8 ± 2.9 (3)	40.2 ± 1.2‡ (3)	19.5 ± 1.7* (5)
+ Ascorbic acid (0.1 mM) and Fe^{2+} (5 μM)	46.8 ± 4.5‡ (3)	20.4 ± 1.9 (3)	50.5 ± 6.5‡ (3)	17.1 ± 2.5‡ (5)

Liver microsomes (0.2 mg protein/mL) in each peroxidation system were incubated at 37° for 1 hr. (A) Fluorescence emitted from the microsomes; (B) Fluorescence emitted from the chloroform/ethanol extracts. Values are means ± SEM; the numbers in parentheses indicate the number of experiments.
* Significantly different from the control ($P < 0.001$).
† Significantly different from the control ($P < 0.05$).
‡ Significantly different from the control ($P < 0.01$).

because of the fluorescence disturbance mentioned above. On the other hand, fluorescent substances were observed in the pellets on ascorbic acid/Fe^{2+}-induced lipid peroxidation of rat liver microsomes, but not in the supernatants [7]. This suggests that fluorescent substances formed during Adriamycin-induced lipid peroxidation remain in microsomes. Fluorescent substances have been determined to be chromolipids extracted with organic solvents from peroxidized membranes [8–10]. In addition to fluorescent lipids, membrane proteins have been found to be another important component of fluorescent substances [15]. The direct detection of fluorescence from peroxidized membranes can give information on both fluorescent lipids and proteins, simultaneously. In an earlier work, we succeeded in determining the fluorescence lifetimes of fluorescent substances in ascorbic acid/Fe^{2+}-peroxidized micro-

somes [16]. Thus, the analysis of fluorescent substances by detecting the fluorescence directly can be of great use in characterizing Adriamycin-induced lipid peroxidation.

The formation of high molecular weight protein aggregates during lipid peroxidation has been observed in liver microsomes [9] and hepatocytes [10] treated with ascorbic acid/Fe^{2+}, ADP/Fe^{3+} or cumen hyderoperoxide. These works showed a qualitative formation of high molecular weight protein aggregates on lipid peroxidation induced by typical peroxidation reaction systems, but did not show whether lipid peroxidation through the drug oxidation by drug-metabolizing enzymes could produce such aggregation. The electrophoretic patterns in Fig. 5 showed clearly that Adriamycin in the liver microsomal oxidation system enhanced the production of high molecular weight protein aggregates. The formation of high molecular weight protein aggregates showed a time-dependent increase (Fig. 6). The high molecular weight protein aggregates formed during Adriamycin-induced lipid peroxidation were shown to remain in the microsomal membranes (Fig. 7B). On the other hand, TBA-RS, after forming in the membranes, was for the most part transferred from the membranes to the medium (Fig. 7A). This result suggests that TBA-RS, after formation in tissues during *in vivo* Adriamycin-induced lipid peroxidation, disappears from the reaction site and may readily be excreted, because TBA-RS is water soluble.

TBA-RS is a good indicator for the general evaluation of lipid peroxidation. On the other hand, fluorescent substances and high molecular weight protein aggregates, which are reliable indicators in the evaluation of Adriamycin-induced lipid peroxidation as shown in Table 1, can be of great use to specify the tissue and the area in the tissue where lipid peroxidation occurs *in vivo*, because such indicators remain in the tissues.

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