Effects of Diffusible Products of Peroxidation of Rat Liver Microsomal Lipids

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The effects on cellular structures of products of peroxidation of rat liver microsomal lipids were investigated. A system containing actively peroxidizing liver microsomal fraction was separated from a revealing or target system by a dialysis membrane. The target system, contained in the dialysis tube, consisted of either intact cells (erythrocytes) or subcellular fractions (liver microsomal fraction). When liver microsomal fractions were incubated with NADPH (or an NADPH-generating system), lipid peroxidation, as measured by the amount of malonaldehyde formed, occurred very rapidly. The malonaldehyde concentration tended to equilibrate across the dialysis membrane. When the target system consisted of erythrocytes, haemolysis occurred abruptly after a lag phase. The lysis was greatly accelerated when erythrocytes from vitamin E-deficient rats were used, but no haemolysis was observed when erythrocytes from vitamin E-treated rats were used. When, in the same system, freshly prepared liver microsomal fractions were exposed to diffusible factors produced by lipid peroxidation, the glucose 6-phosphatase activity markedly decreased. A similar decrease in glucose 6-phosphatase activity, as well as a smaller but significant decrease in cytochrome *P*-450, was observed when the target microsomal fractions were exposed to diffusible factors derived from the peroxidation of liver microsomal lipids in a separate preincubation step. These and additional experiments indicated that the toxicological activity is relatively stable. Experiments in which the hepatic microsomal fractions destined for lipid peroxidation contained radioactively labelled arachidonic acid, previously incorporated into the membranes, showed that part of the radioactivity released from the microsomal fraction into the incubation medium entered the dialysis tube and was recovered bound to the constituents of the microsomal fractions of the target system. These results indicate that during the course of the peroxidation of liver microsomal lipids toxic products are formed that are able to induce pathological effects at distant loci.

Peroxidation of unsaturated lipids, initially studied in the chemistry of oil and fat rancidity, has been subsequently widely investigated in the biological field because of a suspected role in a variety of pathological conditions (Mengel, 1968; Slater, 1972; Plaa & Witscki, 1976), and particularly in liver injury induced by carbon tetrachloride and other halogenoalkanes (Comporti *et al.*, 1965; Recknagel & Ghoshal, 1966; Slater & Sawyer, 1971; Reynolds, 1972). The pathological consequences of lipid peroxidation at the cellular level have usually been considered to be associated with alterations of lipoprotein membranes. However, another way in which it may produce its damaging effects has emerged from studies (Reiner *et al.*, 1972; Uehleke *et al.*, 1973; Glende *et al.*, 1976; Benedetti *et al.*, 1977b) that have suggested that products originating from the peroxidative breakdown of membrane fatty acids are capable of inducing functional

damage in the cell. This possibility is supported by the results of several lines of research. Some studies (Glende *et al.*, 1976; Benedetti *et al.*, 1977b) have shown that the lipid-peroxidation-induced inhibition of certain enzyme activities bound to the membranes of liver endoplasmic reticulum, such as glucose 6-phosphatase and cytochrome *P*-450, occurs only when an interaction of unsaturated fatty acids with O₂ has taken place. Another line of research has been concerned with the effects of the environment created by lipid peroxidation on added intact cells as a revealing system. It is known (Hochstein & Ernster, 1963; Orrenius *et al.*, 1964) that NADPH oxidation by liver microsomal fraction in the absence of electron-accepting terminal substrate results in peroxidation of microsomal lipids. The addition of erythrocytes to this NADPH/microsomal system results in an almost complete haemolysis (Hochstein, 1966). Pfeifer & McCay (1971) suggested that the

lysis of erythrocytes is due to some free-radical species (in their view, probably a hydroxyl radical) originating from the microsomal mixed-function oxidase system and capable of attacking the erythrocyte membrane. On the other hand, Roders *et al.* (1977, 1978) have presented convincing evidence that the factor that causes the lysis of erythrocytes arises from the peroxidation of microsomal lipids. Roders *et al.* (1977) have shown that erythrocytes added to a filtrate of peroxidized liver microsomal fraction suffer prelytic damage, which can be detected by an osmotic-fragility test. It was, moreover, observed (Recknagel & Turocy, 1977) that products derived from peroxidized microsomal fraction in essentially the same conditions that cause haemolysis are highly toxic for cells (the protozoan *Paramecium multimicronucleatum*) phylogenetically unrelated to rat erythrocytes.

These experimental results led to the hypothesis that products arising from localized sites of lipid peroxidation in the membrane can move from their point of origin and induce pathological effects at distant loci. In view of the importance of the problem under investigation, experiments were designed in which the system containing actively peroxidizing microsomal fraction was separated from the revealing or target system by a dialysis membrane. The target system consisted of either intact cells (erythrocytes) or subcellular fractions (liver microsomal fraction). The latter were used to detect possible effects of inactivation of membrane-bound enzymes (glucose 6-phosphatase and cytochrome P-450) by peroxidation products.

The present results show that active products derived from the peroxidation of liver microsomal lipids are diffusible and are capable of inducing damage in cells or subcellular fractions of the target system contained within the dialysis tube.

Experimental

Animals

Male Sprague-Dawley rats (200–250g), maintained on a pellet diet (Piccioni, Brescia, Italy) free of preservative compounds, were used. All the animals, except the erythrocyte donors, were starved overnight before being killed. When vitamin E-treated rats were used, a single oral dose (150mg/100g body wt.) of all-*rac*- α -tocopherol acetate (Hoffman-La Roche, Basel, Switzerland), dissolved in a small amount of olive oil, was given to the animals 16h before they were killed. When vitamin E-deficient rats were used, a vitamin E-deficient diet [Piccioni; prepared as described by Evans & Burr (1927), modified as described by Bacharach & Allchorne (1938)] was given to weanling animals. In the experiments with

phenobarbital-treated rats, a daily dose (80mg/kg body wt.) of phenobarbital was given intraperitoneally for 5 days before death. In the experiments with radioactive-arachidonic acid-treated rats, the label was administered as follows: [5,6,8,9,11,12,14,15- ^3H]arachidonic acid (sp. radioactivity 0.32 Ci/mg; NEN Chemicals, Dreieichenheim, Germany) was diluted with non-radioactive arachidonic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) and dissolved, as the potassium salt, in rat serum to which bovine serum albumin (Sigma) had been added (Clouet *et al.*, 1974); 0.6ml of serum (containing 87.80×10^6 d.p.m.) was injected intravenously into the animals (body wt. 198–202g), and 10min later they were killed and the livers removed after perfusion *in situ* with normal rat serum.

Haemolysis experiments

Liver microsomal fraction was prepared from 10% (w/v) liver homogenates in ice-cold 0.09M-NaCl buffered with 0.05M-sodium phosphate, pH 6.6, as previously reported (Benedetti *et al.*, 1977a). The microsomal sediment was appropriately resuspended in the same medium.

Rat erythrocytes (target system) were prepared as previously reported (Benedetti *et al.*, 1977a). The packed erythrocytes were diluted 1:5 with 0.9% NaCl to yield a 20% (v/v) erythrocyte suspension. The suspensions were stored at 4°C for 1 night before being used, except those in which the erythrocytes were derived from vitamin E-deficient rats, in which case considerable haemolysis occurred after a few hours of storage.

Glucose 6-phosphatase and cytochrome P-450 experiments

Liver microsomal fraction destined for lipid peroxidation was prepared from 10% (w/v) liver homogenates in ice-cold 0.15M-KCl buffered with 0.05M-Tris/maleate, pH 6.6. The homogenate was centrifuged at 2700g for 10min. The supernatant fraction was centrifuged at 100000g for 30min. The resulting microsomal pellet was appropriately resuspended in the incubation buffer. In some experiments the microsomal fraction destined for lipid peroxidation was obtained from phenobarbital-treated rats.

In simultaneous incubation experiments (see below) the liver microsomal fraction used as the target system was prepared from vitamin E-treated rats. It was isolated from 10% (w/v) liver homogenates in ice-cold 0.25M-sucrose/6mM-EDTA, pH 7.4, by the method of de Duve *et al.* (1955). The microsomal pellet was resuspended in 0.15M-KCl buffered with 0.05M-Tris/maleate, pH 6.6. Use of

vitamin E-treated rats and isolation in media enriched with EDTA were necessary to prevent lipid peroxidation and consequent loss of the enzyme activities in the microsomal fraction contained within the dialysis tube during the incubation: NADPH can enter the dialysis tube and promote lipid peroxidation of microsomal fraction contained within it, if such microsomal fraction has not been obtained from vitamin E-treated rats and prepared in media free of EDTA. However, if such a microsomal fraction is prepared as prescribed above, it will not peroxidize within the dialysis tube, despite the movement of NADPH into the dialysis tube. Conditions for sequential-type experiments (see below) are given in the legends to pertinent Tables. From previous studies in our laboratory (Benedetti *et al.*, 1974), the concentration of α -tocopherol in the microsomal fraction of rats treated with vitamin E at the dose reported above was $2.3 \mu\text{g}/\text{mg}$ of microsomal protein (the value for untreated rats was $0.2 \mu\text{g}/\text{mg}$ of microsomal protein).

Incubation procedures

The incubation systems and conditions used in the various experiments are reported in full in the legends to the appropriate Tables and to Fig. 1. In all the experiments, the suspension of liver microsomal fraction destined for lipid peroxidation was incubated aerobically in an Erlenmeyer flask in the presence of NADPH or an NADPH-generating system. Samples of the erythrocyte or liver microsomal fraction suspension used as target systems were introduced into a dialysis tube. The dialysis tube was a 25cm section of Visking dialysis tubing 20/32 (Serva Feinbiochemica, Heidelberg, Germany; diameter 16mm; wall strength 0.020mm; average pore diameter 1.5–2.0nm; exclusion limit 10000–20000mol.wt.). The tube was bent into a U-shape, with the upper limbs pressed between the cork and the neck of the Erlenmeyer flask with the U-shaped lower portion (containing the suspension) immersed in the incubation medium. In some experiments (see the Results section) the dialysis tube with the target system was introduced into the Erlenmeyer flask at the beginning of the incubation. Such an incubation procedure is referred to as simultaneous incubation. In other experiments the microsomal fraction destined for lipid peroxidation was first preincubated and allowed to peroxidize without the target system. Lipid peroxidation was then stopped by the addition of EDTA. Afterwards, the dialysis tube containing the target system was introduced into the Erlenmeyer flask and the flask was incubated for an additional 60min. This incubation procedure is referred to as sequential incubation.

In general, three sets of controls were carried out. One (plus NADPH, minus microsomal fraction) was

identical with the experimental flask, except no liver microsomal fraction was added in the Erlenmeyer flask outside the dialysis tube. Another one (plus microsomal fraction, minus NADPH) was identical with the experimental flask except no NADPH (or NADPH-generating system) was added. In the third one (no additions) both microsomal fraction and NADPH (or the NADPH-generating system) were omitted. The latter control was not carried out in some experiments.

Enzyme assays and other analytical procedures

At the end of the incubation, samples of the mixtures contained both outside and inside the dialysis tube were drawn off for malonaldehyde determination. The rest of the contents of the dialysis tube was used for the determination of either the extent of haemolysis, in the haemolysis experiments, or the residual enzyme activities of liver microsomal fraction, in the glucose 6-phosphatase and cytochrome *P*-450 experiments. In the latter case the microsomal fraction contained in the dialysis tube was resedimented by centrifugation at 100000g for 30min, and appropriately resuspended for enzyme assay.

The determination of haemolysis was performed as previously reported (Benedetti *et al.*, 1977a). Glucose 6-phosphatase activity was assayed by the method of de Duve *et al.* (1955). P_i was determined as reported by Harper (1963). The assay for cytochrome *P*-450 content was as described by Omura & Sato (1964). Since Nishibayashi *et al.* (1968) have shown that CO is formed during the NADPH-dependent peroxidation of liver microsomal lipids, the A_{450} of the CO–cytochrome *P*-450 complex in the experimental sample was corrected for A_{450} of the same sample tested against a corresponding suspension of non-peroxidized microsomal fraction. Such a correction was necessary to take into account the net effect of lipid-peroxidation products other than CO on the cytochrome *P*-450 content of the liver microsomal fraction of the target system. The amount of malonaldehyde formed was determined as previously reported (Comporti *et al.*, 1967), with a malonaldehyde standard produced by the acid hydrolysis of 1,1,3,3-tetraethoxypropane. Protein determination was performed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Plasma α -tocopherol content was determined as previously described (Benedetti *et al.*, 1974).

In the experiments in which liver microsomal fraction derived from radioactively labelled arachidonic acid-treated rats was used in the peroxidizing system, at the end of the incubation both the suspension of the peroxidized microsomal fraction and that of the microsomal fraction of the target system were centrifuged at 100000g for 45min. Portions of the supernatant fractions were added to 10ml of

Insta-Gel (Packard Instrument Co., Downers Grove, IL, U.S.A.) and counted for radioactivity in a liquid-scintillation counter (LKB Ultrabeta, Stockholm, Sweden). Samples of the resedimented microsomal fraction were extracted as described by Folch *et al.* (1957). The extracted lipids were dissolved in 10 ml of scintillation fluid [0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene] and counted for radioactivity. The extracted microsomal residue was quantitatively recovered on a glass-fibre paper disc, washed several times with a chloroform/methanol mixture (2:1, v/v) and then extracted again with the same mixture by refluxing at 65°C for 6 h. The non-lipid residue was quantitatively recovered, hydrolysed by adding 0.1 ml of water and 1 ml of Soluene 350 (Packard) and counted for radioactivity after the addition of 10 ml of scintillation fluid. An additional sample of the sediment of peroxidized microsomal fraction was counted for radioactivity after hydrolysis with Soluene 350 (1 ml) and addition of scintillation fluid

(10 ml). The counts were quench-corrected by the channels-ratio method and the background value was subtracted. The counting efficiency ranged between 23 and 47% in the various samples.

Results

Lysis of erythrocytes exposed to the diffusible factors derived from the peroxidation of liver microsomal lipids

Simultaneous-incubation experiments were performed. As shown in Figs. 1(a)–1(c), the incubation of liver microsomal fraction with NADPH resulted in a rapid onset of lipid peroxidation, as measured by the amount of malonaldehyde produced. The malonaldehyde concentration outside and inside the dialysis tube tended to equilibrate. When erythrocytes from rats maintained on the stock diet were used as target system (Fig. 1a), haemolysis started after a lag phase of 30 min and proceeded up to its maximal value (about 80%) between 90 and 120 min

Fig. 1. *Lysis of erythrocytes exposed to diffusible factors derived from the peroxidation of liver microsomal lipids*
Erythrocytes derived from rats maintained on the stock diet (a), vitamin E-deficient rats (b) and vitamin E-treated rats (c) were used. The experimental flask (upper graphs) contained, in a volume of 22 ml, 1 g-equiv. of microsomal fraction, 0.09 M-NaCl, 0.05 M-sodium phosphate buffer, pH 6.6, and 0.9 mM-NADPH. Control flasks were identical with the experimental flask, except that no microsomal fraction or no NADPH was added. A dialysis tube containing erythrocytes [0.2 ml of a 20% (v/v) suspension in 0.9% NaCl] and 1.8 ml of 0.09 M-NaCl, 0.05 M-sodium phosphate buffer, pH 6.6, was introduced into the flasks at the start of the incubation (simultaneous incubation). The incubation was carried out at 37°C. At the incubation times indicated, the concentration of malonaldehyde, both outside and inside the dialysis tube, as well as the percentage haemolysis, were determined. Points are means $\pm$ S.E.M. (vertical bars) for three experiments. Points without S.E.M. are the means for two experiments. Symbols: ○, malonaldehyde outside dialysis tube; Δ, malonaldehyde inside dialysis tube; ●, haemolysis. (a) (i)–(c) (i), Experimental results; (a) (ii)–(c) (ii), control (minus microsomal fraction) results; (a) (iii)–(c) (iii), control (minus NADPH) results.

of incubation. This result confirmed our previous findings (Benedetti *et al.*, 1977a).

Since it is known that in vitamin E deficiency a lower stability of erythrocytes occurs (Rose & Gyorgy, 1952; Marvin, 1963; Bieri & Poukka, 1970; Barker *et al.*, 1973), erythrocytes from either vitamin E-deficient or vitamin E-treated rats were tested for haemolysis in the incubation system just described. It was observed that the haemolysis is markedly accelerated in the erythrocytes from vitamin E-deficient rats (Fig. 1b) compared with that occurring in erythrocytes from rats maintained on the stock diet (Fig. 1a). The lysis was in fact almost maximal at 60 min of incubation (the difference between the 60 min values for haemolysis in erythrocytes from rats maintained on the stock diet and those from vitamin E-deficient rats was statistically significant at $P < 0.001$). At 120 min, extensive haemolysis also occurred in the control samples (Fig. 1b). On the other hand, in the erythrocytes from vitamin E-treated rats (Fig. 1c), the lysis was minimal even at 120 min of incubation (the difference between the 120 min values for haemolysis in erythrocytes from rats maintained on the stock diet and those from vitamin E-treated rats was statistically significant at $P < 0.001$). Plasma α -tocopherol concentrations were as follows: in rats maintained on the stock diet, 1.3 ± 0.2 (mean $\pm$ S.D.) $\mu\text{g/ml}$; in rats maintained on

vitamin E-deficient diet, 0 in most cases (0.01 and 0.16 in two cases); in rats treated with vitamin E, $11.9 \mu\text{g/ml}$.

Loss of glucose 6-phosphatase activity and of cytochrome P-450 of liver microsomal fraction exposed to diffusible factors derived from the peroxidation of liver microsomal lipids

The effects of the diffusible factors produced by NADPH-dependent peroxidation of liver microsomal lipids on enzyme activities bound to the membranes of the liver endoplasmic reticulum were investigated. When liver microsomal fraction was incubated along with the target system, i.e. under simultaneous-incubation conditions, a vigorous production of malonaldehyde ensued (Table 1). Essentially similar malonaldehyde concentrations were found both outside and inside the dialysis tube. After 120 min of incubation, glucose 6-phosphatase activity of the liver microsomal fraction of the target system was markedly (about 45%) decreased in the experimental samples compared with the controls, in which little malonaldehyde production occurred. A smaller (about 30%), although significant, decrease in enzyme activity was observed at 60 min of incubation.

The importance of the above observations is emphasized by the results of additional experiments

Table 1. *Loss of glucose 6-phosphatase activity in liver microsomal fraction exposed to diffusible factors produced by peroxidation of liver microsomal lipids*

The experimental flask [+NADPH-generating system (indicated as NADPH) + microsomal fraction] contained, in a final volume of 22 ml, 1 g-equiv. of liver microsomal fraction (derived from phenobarbital-treated rats), 0.15 M-KCl, 0.05 M-Tris/maleate buffer, pH 6.6 (in the case of experiments involving glucose 6-phosphatase, since ultimate analysis for the enzyme involves measurement of P_i , the buffer medium cannot contain P_i ; it is for this reason that the KCl/Tris/maleate buffer was employed) and an NADPH-generating system [0.1 mM-NADP⁺, 20 mM-DL-isocitrate, 5.0 mM-MgCl₂, 2.5 mM-nicotinamide and 0.1 mg of isocitrate dehydrogenase (Boehringer G.m.b.H., Mannheim, Germany; specific activity approx. 2 units/mg of protein per ml). One unit of enzyme activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol of substrate/min at 25°C under optimal conditions. FeSO₄ (0.01 mM final concentration) was added to enhance lipid peroxidation. Control flasks were identical with the experimental flask, except that no microsomal fraction or no NADPH-generating system (plus FeSO₄) was added, or neither were added. A dialysis tube containing 2.0 ml of a suspension of 100 mg-equiv. of liver microsomal fraction (derived from vitamin E-treated rats) in the KCl/Tris/maleate buffer was introduced into the flasks at the start of incubation (simultaneous incubation). The incubation was carried out at 38°C. At 60 and 120 min of incubation the concentration of malonaldehyde both outside and inside the dialysis tube and the glucose 6-phosphatase activity of the microsomal fraction contained in the dialysis tube were evaluated. Results are means $\pm$ S.E.M. for three experiments.

Incubation time (h)	Conditions	Malonaldehyde concn. ($\mu\text{g/ml}$)		Glucose 6-phosphatase activity (μmol of P_i /20 min per mg of protein)	Variation (%)
		Outside	Inside		
1	No addition	0.03 ± 0.006	0.03 ± 0.005	9.63 ± 0.46	—
1	+NADPH—microsomal fraction	0.03 ± 0.001	0.04 ± 0.008	9.71 ± 0.44	+0.8
1	+microsomal fraction—NADPH	0.63 ± 0.15	0.52 ± 0.18	9.65 ± 0.31	+0.2
1	+microsomal fraction+NADPH	6.75 ± 1.08	6.16 ± 0.97	6.69 ± 0.53	-30.5
2	No addition	0.02 ± 0.003	0.01 ± 0.008	9.48 ± 0.75	—
2	+NADPH—microsomal fraction	0.03 ± 0.018	0.03 ± 0.011	8.75 ± 0.38	-7.7
2	+microsomal fraction—NADPH	1.56 ± 0.05	1.22 ± 0.09	8.27 ± 0.14	-12.8
2	+microsomal fraction+NADPH	6.94 ± 0.85	6.27 ± 0.61	5.00 ± 0.39	-47.3

in which sequential incubation was followed. Table 2 shows that glucose 6-phosphatase activity of liver microsomal fraction was decreased (43%) when the microsomal fraction was exposed to the diffusible factors derived from the peroxidation of liver microsomal lipids occurring in a separate preincubation step. The decrease in enzyme activity observed after the post-incubation step was similar to that found in the simultaneous-incubation experiments. It was also observed (Table 3) that the amount of cytochrome *P*-450 of the microsomal fraction of the target system was significantly decreased in similar sequential-incubation experiments. It is therefore possible to deduce that toxic products originating from the peroxidation of liver microsomal lipids during the preincubation step have a sufficiently long life span to bring about damaging effects on the enzyme activities during the

second incubation. Experiments were then carried out to investigate whether the toxic factors derived from lipid peroxidation could be recovered in the supernatant fraction obtained by centrifuging (100000g for 30min) the preincubation mixture (to which 6mM-EDTA was added) at the end of the preincubation. To this purpose, supernatant fractions free of microsomal fraction were incubated with the dialysis tube containing freshly prepared liver microsomal fraction, either immediately or after aging of the supernatant fractions for 48 h at 0–4°C. It was observed that glucose 6-phosphatase activity of the microsomal fraction contained in the dialysis tube is decreased by 44% when the incubation was carried out with non-aged supernatant fraction. The decrement was, however, much lower (about 16%) when the aged supernatant fraction was used.

Additional experiments were carried out to

Table 2. *Loss of glucose 6-phosphatase activity in liver microsomal fraction exposed to diffusible factors derived from microsomal lipid peroxidation occurring during a preincubation step*

The experimental flask (+NADPH, +microsomal fraction) contained, in a final volume of 22ml, 1 g-equiv. of microsomal fraction (derived from normal rats), 0.15M-KCl, 0.05M-Tris/maleate buffer, pH 6.6, and 0.9mM-NADPH. FeSO₄ (0.1mM final concentration) was added to enhance lipid peroxidation. Control flasks were identical with the experimental flask, except that no microsomal fraction or no NADPH/FeSO₄ was added, or neither were added. The flasks were preincubated for 30min at 37°C. After taking samples for malonaldehyde determination, EDTA (6mM) was added to the flasks. A dialysis tube containing 2.0ml of a suspension of 100mg-equiv. of liver microsomal fraction (derived from normal rats) in 0.15M-KCl, 0.05M-Tris/maleate buffer, 6mM-EDTA, pH 6.6, was then introduced into the flasks. The flasks were then incubated for an additional 60min at 37°C. At the end of the post-incubation period the malonaldehyde concentration both outside and inside the dialysis tube and the glucose 6-phosphatase activity of the microsomal fraction contained in the dialysis tube were evaluated. Results are means $\pm$ S.E.M. for three experiments.

Conditions	Malonaldehyde concn. ($\mu\text{g/ml}$)			Glucose 6-phosphatase activity (μmol of P_i /20min per mg of protein)	Variation (%)
	After preincubation	After post-incubation			
		Outside	Inside		
No addition	—	0	0.03 ± 0.01	9.06 ± 0.21	—
+NADPH—microsomal fraction	—	0.03 ± 0.01	0.03 ± 0.01	8.69 ± 0.12	−4.1
+microsomal fraction—NADPH	0.33 ± 0.02	0.62 ± 0.05	0.51 ± 0.05	9.27 ± 0.25	+2.3
+microsomal fraction+NADPH	9.23 ± 0.13	7.27 ± 0.18	6.78 ± 0.07	5.20 ± 0.34	−42.6

Table 3. *Loss of cytochrome P-450 of liver microsomal fraction exposed to diffusible factors derived from microsomal lipid peroxidation occurring during a preincubation step*

Conditions were as described in Table 2. Results are means $\pm$ S.E.M. for three experiments. Statistical significance: (d) against (a), $P < 0.001$; (d) against (b), $P < 0.01$; (d) against (c), $P < 0.05$.

Conditions	Malonaldehyde concn. ($\mu\text{g/ml}$)			Cytochrome <i>P</i> -450 (nmol/mg of protein)	Variation (%)
	After preincubation	After postincubation			
		Outside	Inside		
No addition	—	0	0.03 ± 0.01	1.18 ± 0.018 (a)	—
+NADPH—microsomal fraction	—	0.03 ± 0.01	0.05 ± 0.003	1.17 ± 0.024 (b)	—0.9
+microsomal fraction—NADPH	0.34 ± 0.02	0.70 ± 0.02	0.52 ± 0.02	1.08 ± 0.044 (c)	—8.5
+microsomal fraction+NADPH	9.37 ± 0.32	7.77 ± 0.30	7.13 ± 0.53	0.94 ± 0.021 (d)	—20.3

Table 4. Release of radioactivity from liver microsomal fraction derived from [³H]arachidonic acid-treated rats and incubated in the NADPH-dependent system, binding of the diffusible radioactivity to liver microsomal fraction (target system) contained within the dialysis tube and concomitant loss of glucose 6-phosphatase activity of target microsomal fraction

Conditions were as those reported in Table 2, except that the liver microsomal fraction destined for lipid peroxidation was derived from [³H]arachidonic acid-treated rats (see the Experimental section). Zero-time sample was identical with the experimental flask, except EDTA (6 mM) was added at the beginning of the preincubation and the preincubation was carried out at 0°C; after the dialysis tube containing the target microsomal fraction was introduced into the flask, the sample was incubated at 37°C for 60 min as the experimental flask. At the end of the post-incubation, the suspension of peroxidized microsomal fraction was centrifuged at 100 000*g* for 45 min and the radioactivity was determined in both the microsomal sediment and the supernatant (incubation medium outside the dialysis tube). In addition, the radioactivity present in the lipids and in the non-lipid residue of the microsomal sediment was evaluated. The suspension of the microsomal fraction contained in the dialysis tube (target microsomal fraction) was centrifuged as above and the radioactivity was determined in the supernatant (incubation medium inside the dialysis tube) and in the lipid and non-lipid constituents of the resedimented microsomal fraction. A sample of the resedimented microsomal fraction (target microsomal fraction) was tested for glucose 6-phosphatase activity. After the preincubation, the malonaldehyde concentration in the experimental sample was 9.76 μg/ml of incubation mixture. After the post-incubation, the malonaldehyde concentration was 8.65 and 7.76 μg/ml outside and inside the dialysis tube respectively.

Conditions	10 ⁻³ × Radioactivity recovered in the microsomal sediment (d.p.m./g-equiv. of microsomal fraction)			10 ⁻³ × Radio- activity released into the incubation medium (d.p.m./g-equiv. of microsomal fraction)		10 ⁻³ × Radioactivity present in the target microsomal fraction (d.p.m./g-equiv. of microsomal fraction)		Glucose 6-phosphatase activity in the target microsomal fraction (μmol of P _i /20 min per mg of protein)
	Microsomal fraction	Microsomal lipids	Non-lipid residue of microsomal fraction	Outside	Inside	Total lipids	Non-lipid residue	
Zero-time sample	1157.7	1066.0	13.7	100.4	19.8	1.69	0.25	9.96
+ microsomal fraction – NADPH	1112.9	971.1	16.7	122.3	32.1	2.08	0.24	9.21
+ microsomal fraction + NADPH	796.0	572.7	68.4	509.7	285.5	5.66	6.25	5.92

Conditions were as those reported in Table 2, except that the liver microsomal fraction destined for lipid peroxidation was derived from [³H]arachidonic acid-treated rats (see the Experimental section). Zero-time sample was identical with the experimental flask, except EDTA (6mM) was added at the beginning of the preincubation and the preincubation was carried out at 0°C; after the dialysis tube containing the target microsomal fraction was introduced into the flask, the sample was incubated at 37°C for 60 min as the experimental flask. At the end of the post-incubation, the suspension of peroxidized microsomal fraction was centrifuged at 100000g for 45 min and the radioactivity was determined in both the microsomal sediment and the supernatant (incubation medium outside the dialysis tube). In addition, the radioactivity present in the lipids and in the non-lipid residue of the microsomal sediment was evaluated. The suspension of the microsomal fraction contained in the dialysis tube (target microsomal fraction) was centrifuged as above and the radioactivity was determined in the supernatant (incubation medium inside the dialysis tube) and in the lipid and non-lipid constituents of the resedimented microsomal fraction. A sample of the resedimented microsomal fraction (target microsomal fraction) was tested for glucose 6-phosphatase activity. After the preincubation, the malonaldehyde concentration in the experimental sample was 9.76 μg/ml of incubation mixture. After the post-incubation, the malonaldehyde concentration was 8.65 and 7.76 μg/ml outside and inside the dialysis tube respectively.

investigate whether variations in the amount of the target structures available for the toxic factors originating from lipid peroxidation could result in variations of the apparent effects. It was observed that when the amount of the target microsomal fraction was doubled, the decrease in glucose 6-phosphatase activity is somewhat lower than that observed when the usual amount of microsomal fraction was employed. The sequential-incubation procedure was used in these experiments. An attempt was made to investigate whether the addition of albumin to the microsomal suspension contained in the dialysis tube could result in some protection of glucose 6-phosphatase activity by a shielding effect. It was found that the addition of 100mg of albumin to the microsomal suspension had no significant protective effect.

Binding of diffusible factors derived from the peroxidation of liver microsomal lipids to the microsomal fraction contained within the dialysis tube

Previous studies from our laboratory (Benedetti *et al.*, 1977c) have shown that when liver microsomal fraction into which radioactively labelled arachidonic acid had been incorporated are peroxidized in the NADPH-dependent system, part of the radioactivity is released from the microsomal fraction into the incubation medium, and part is recovered bound to the microsomal protein. It was therefore investigated whether some of the radioactivity released into the incubation medium could cross the dialysis membrane and bind to the microsomal fraction of the target system contained within the dialysis tube. To this end the liver microsomal fraction destined for lipid peroxidation was obtained from rats injected with [^3H]arachidonic acid and killed 10min later (see the Experimental section). Benedetti *et al.* (1977c) have shown that over 96% of the radioactivity present in the fatty acids of phospholipids and over 91% of that present in the fatty acids of neutral lipids of microsomal fractions obtained in such experiments is associated with arachidonic acid. This means that no metabolic recycling of the radioactivity associated with arachidonic acid into the other fatty acids occurs within 10min of the injection. When liver microsomal fraction prepared under such experimental conditions was used in the peroxidizing system (Table 4), a marked production of malonaldehyde was found at the end of the preincubation period (the sequential-incubation procedure was followed in these experiments). The malonaldehyde concentration was slightly lower both outside and inside the dialysis tube at the end of the post-incubation period. As shown in Table 4, there was a consistent release of radioactivity from the peroxidizing microsomal fraction to the incubation medium, that is the supernatant fraction obtained by centrifuging the incubation mixture at the end of the

postincubation period. In agreement with our previous results (Benedetti *et al.*, 1977c), some of the radioactivity lost by the microsomal lipids was found to be bound to the non-lipid constituents of the peroxidized microsomal fraction (Table 4). Part of the radioactivity released from the microsomal fraction into the incubation medium crossed the dialysis membrane and was found in the supernatant fraction obtained by centrifuging the microsomal suspension contained within the dialysis tube (Table 4). Some of the radioactivity that entered the dialysis tube was recovered in the lipid and non-lipid constituents of the microsomal fraction of the target system (Table 4). The radioactivity found in the lipid constituents could be, at least in part, adsorbed on the target microsomal fraction, whereas the radioactivity found in the non-lipid constituents was very likely covalently bound. Concomitantly with the binding of radioactive products derived from lipid peroxidation, a decrease in glucose 6-phosphatase activity in the target microsomal fraction was observed (Table 4).

Similar results were obtained in additional experiments (not reported) in which simultaneous incubation was carried out.

Discussion

The present results indicate that during the course of peroxidation of liver microsomal lipids, toxic products are formed that have the capacity to induce pathological effects at distant loci. The effects are detectable in systems that are separated by a dialysis membrane from the system in which lipid peroxidation occurs. Thus the toxic products are of a relatively small molecular size. They can bring about damaging effects on cellular membranes, as shown by the lytic activity on erythrocytes, as well as on enzymic molecular complexes located in the membranes, as shown by the partial inactivation of glucose 6-phosphatase and cytochrome *P*-450. The impairment of glucose 6-phosphatase is rather extensive, whereas that of cytochrome *P*-450 is less so. It is noteworthy, however, that glucose 6-phosphatase was found to be more labile to lipid peroxidation than was cytochrome *P*-450, even in non-compartmentalized systems, i.e. when the enzyme activities were tested in the microsomal fraction itself, which underwent lipid peroxidation (Glende *et al.*, 1976).

The active factors do not seem to be of a free-radical nature for the following reasons. (i) It seems quite unlikely that any free radical with sufficient reactivity would be able to move from the microsomal fraction, traverse the aqueous medium as well as the wall of the dialysis tube, and attack the target structures contained inside it. The very high reactivity as well as the extremely short half-life of transient

free radicals formed by metabolic activation seems to preclude any possibility that free-radical species originating during the NADPH oxidation are the agents responsible for the damaging effects on the target structures contained in the dialysis tube. (ii) The results obtained in the subsequent-incubation experiments, as well as the fact that the toxicological activity can survive the centrifugation of the pre-incubation mixture, clearly indicate that the active products originating during the preincubation step have a sufficiently long life span to impair the enzyme activities of freshly prepared liver microsomal fraction during the second incubation. Such long-lasting toxicological activity is not compatible with reactive free-radical species. (iii) Finally, the results (Table 4) of experiments in which the hepatic microsomal fraction destined for lipid peroxidation contains radioactively labelled arachidonic acid, previously incorporated into the membranes, suggest that an interaction occurs between products originating from the peroxidative breakdown of unsaturated lipids and the microsomal fraction of the target system. Interactions of products derived from lipid peroxidation with cellular constituents have been extensively reported (Roubal & Tappel, 1966; Chio & Tappel, 1969*a,b*; Bidlack & Tappel, 1973).

In similar experiments, but carried out in non-compartmentalized systems, lysis of erythrocytes derived from anti-oxidant-treated rats was not detected by McCay *et al.* (1971). This result was explained by postulating a scavenging effect of anti-oxidants in the erythrocyte membrane against some free-radical species arising from the peroxidizing microsomal fraction. The present work provides an alternative explanation for the observation of McCay *et al.* (1971). Our results indicate that the active entity arising from the peroxidizing microsomal fraction is not a free radical. However, the attack by the active entity on erythrocyte membranes may involve radical mechanisms. For example, if the active haemolytic entity is a lipoperoxide, its cleavage at the level of the erythrocyte may initiate peroxidative decomposition of lipids in the erythrocyte membrane. Such a conception allows for the observed resistance of vitamin E-enriched cells to haemolysis in the microsomal fraction/NADPH system and the increased sensitivity of erythrocytes from vitamin E-deficient rats, and it encompasses the key results of the present paper, which indicate that the active entity traversing the gap between peroxidizing microsomal fraction and the target erythrocytes is not itself a free radical.

When the effect on glucose 6-phosphatase activity of the diffusible factors produced during the incubation of liver microsomal fraction was investigated, it was found (simultaneous-incubation experiments) that high concentrations of tocopherol in the microsomal fraction of the target system did not prevent

inactivation of the enzyme. This result suggests that the attack on glucose 6-phosphatase may involve mechanisms different from those involved in the attack on erythrocytes.

From the present results it appears that the active factors generated during the incubation of liver microsomal fraction are products originating from the peroxidation of liver microsomal lipids. As shown by Roders *et al.* (1978), malonaldehyde, which in the present work was taken as an index of lipid peroxidation, does not appear to be the haemolytic factor. Malonaldehyde, directly added to erythrocytes at concentrations much higher than that produced during the incubation of liver microsomal fraction, had no haemolytic effect (Roders *et al.*, 1978). The results of similar experiments (Roders *et al.*, 1978) also excluded H_2O_2 , a known haemolytic agent that has been detected in microsomal systems during NADPH oxidation (Boveris *et al.*, 1972; Thurman *et al.*, 1972) as the haemolytic factor. Experiments carried out in our laboratory have shown that malonaldehyde, at concentrations similar or 5 to 10 times higher than those of malonaldehyde, formed in the experimental flask during the incubation, has no inhibitory effect on microsomal glucose 6-phosphatase activity.

The characteristics of the dialysis membrane (10000–20000 mol.wt. exclusion limit) make it highly unlikely that the active factors are functional parts of the microsomal electron-transport system, possibly released from the microsomal fraction during lipid peroxidation. Rather, the pore size of the dialysis membrane allows the transit of lipid-peroxidation products such as lipoperoxides, aldehydes, ketones and polymerization or condensation compounds of relatively small molecular size. Lipoperoxides, for instance, may initiate further lipid peroxidation at remote sites (Shimasaki & Privett, 1975; Sies & Summer, 1975) and may thus cause loss of membrane structure in erythrocytes with subsequent lytic damage. Aldehydes, on the other hand, may covalently bind to active sites of enzymes (Chio & Tappel, 1969*b*; Shin *et al.*, 1972; Bertone & Dianzani, 1977).

The studies by Wills (1971) on the relationship between the peroxidation of liver microsomal lipids and the inactivation of microsomal glucose 6-phosphatase activity led to the conclusion that the loss of activity of glucose 6-phosphatase is a direct consequence of loss of membrane structure essential for the enzyme activity and that no products toxic to the enzyme are formed during lipid peroxidation. In these studies suspensions of peroxidized microsomal fraction were mixed with suspensions of non-peroxidized microsomal fraction and the glucose 6-phosphatase activity was measured immediately after mixing; no inhibition of the enzyme activity was found. The present work shows that a relatively long

time interval must elapse before the active products originating from lipid peroxidation induce haemolysis (in erythrocytes from rats maintained on the stock diet) or enzyme inhibition. The lack of inhibition found in the studies by Wills (1971) may thus be explained. The time interval that must elapse before haemolysis or enzyme inhibition occurs may represent the time required for the active factors to induce secondary peroxidation in the membranes of the target structures. Alternatively, a threshold concentration of the active factors may have to be reached in the dialysis tube for the damaging effects to take place.

In conclusion the present work shows that toxic products or their proximate precursors originating from the peroxidation of liver microsomal lipids are able to move from their site of origin (the lipid core of the membrane) and cover tremendous distances, in molecular terms, such as that occurring between the microsomal fraction and the target systems contained within the dialysis tube. Furthermore the compartmentalized system reported in the present paper may represent an experimental device suitable for future studies to characterize the toxic factors.

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