quence has been shown to induce delayed type hypersensitivity (*12*) unaccompanied by clinical or histological signs of EAE; however, the encephalitogenic potency and species susceptibility to this region of the myelin BP need further definition.

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8. Abbreviations: Ala, alanine; Arg, argine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Leu, leucine; Lys, lysine; Pro, proline; and Ser, serine.
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## Prelytic Damage of Red Cells in Filtrates from Peroxidizing Microsomes

Abstract. *When liver microsomes are incubated in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), their constituent lipids undergo peroxidative degeneration. If erythrocytes are present in such a peroxidizing system, they hemolyze. Filtrates obtained by ultrafiltration of peroxidizing microsomal systems were found to have the capacity to produce prelytic damage in red cells. Filtrates obtained from microsomes that had not undergone peroxidative lipid decomposition were inert. The toxic activity in the active filtrates was not due to continuing oxidation of NADPH nor to continuing liver microsomal lipid peroxidation. Neither the chemical identity of the toxic product or products in active filtrates nor the mechanisms involved in the erythrocyte damage are known at this time.*

Peroxidation of lipids in biological membranes is an abnormal and destructive phenomenon. It has been implicated in a variety of pathophysiological conditions that often, although not always, arise from toxigenic sources (*1–10*). If one assumes that lipid peroxidation is a critical vector in a particular condition, the question remains as to the exact mechanisms whereby the pathological involvement of the structure and function of the cell as a whole can result from a process that, at least initially, occurs at a circumscribed locus. In other words, if lipid peroxidation is induced at one location within a cell, for example, the cytochrome P450 locus of the endoplasmic reticulum, what are the critical events which eventually result in destructive manifestations at other locations? One possibility is that toxic metabolites arising from a particular site have the capacity of inducing pathological effects elsewhere in the cell. We use erythrocytes as a biological device to give evidence that such toxic metabolites of lipid peroxidation most likely exist.

When liver microsomes are incubated in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), without any exogenous electron accepting substrate, lipid peroxidation occurs (*11*). It has been reported that when erythrocytes are added to a peroxidizing microsomal mixture they hemolyze (*12*). Given the generally deleterious effects with which lipid peroxidation is associated, it is possible that the agent responsible for the red cell lysis is a product of this microsomal peroxidation. Supportive of this idea would be the recent finding that erythrocytes incubated in the presence of linoleic hydroperoxide undergo hemolysis (*13*). However, when this phenomenon was previously investigated, the conclusion was reached that microsomal lipid peroxidation was in no way responsible for the erythrocytic hemolysis (*14*). Rather, it was concluded that a free radical species (most likely hydroxyl radical) emerged from the microsomal electron transport system and directly induced the hemolysis. On reinvestigating this phenomenon we have obtained evidence that microsomal lipid peroxidation plays a more critical role in the hemolysis than previous investigators suspected.

Among the experiments that suggest that microsomal lipid peroxidation may be the decisive event expressed ultimately in the erythrocytic damage are the following. When malonic dialdehyde (MDA) production and hemolysis are monitored in the same incubation mixture, MDA appears well before the red cells lyse (Fig. 1). Since the appearance of MDA is used to infer that microsomal lipid peroxidation has occurred, this particular sequence of events is consistent with the idea that some product arising from the peroxidation process in the microsomal membrane is responsible for the subsequently occurring erythrocytic damage. MDA is most certainly not the hemolytic agent since, when it is added to erythrocytes at concentrations one hundred times in excess of what is observed in Fig. 1, no hemolysis can be detected.

Furthermore, no hemolysis occurs when red cells are incubated in the presence of microsomes, NADPH, and the terminal substrate aminopyrine. Active electron transport occurs in this system as evidenced by production of formaldehyde; however, lipid peroxidation does not occur in presence of the substrate. Thus, mixed function oxidase activity per se is insufficient to induce erythrocytic damage; rather, peroxidation of microsomal lipids seems to be the requisite precondition for subsequently occurring red cell hemolysis.

These experiments led us to carry out much more critical experiments. These experiments have demonstrated that filtrates derived from peroxidizing microsomes retain the capacity to produce prelytic damage in red cells. This phase of our work was facilitated by use of microsomes recovered from calcium-rich media (*15*). These aggregated calcium

microsomes readily undergo peroxidation in the presence of NADPH and are retained by membrane-type filters. If calcium microsomes supplemented with NADPH are incubated for 10 minutes, lipid peroxidation can be detected. Rapid ultrafiltration of this mixture yields a filtrate essentially free of microsomes. Addition of EDTA and erythrocytes to this filtrate, followed by a subsequent 60-minute incubation, results in extensive prelytic damage to the red cells. This damage can be detected by use of an osmotic fragility test conducted on the recovered erythrocytes. Red cells that have been exposed to the filtrate from peroxidized microsomes show greatly increased osmotic fragility (Fig. 2). Conversely, addition of red cells to the filtrate of nonperoxidized microsomes, with added EDTA, results in no evidence of damage even if the filtrate is supplemented with NADPH. Free radicals are very unstable and short-lived. It is unlikely that such a transient species would survive the filtration process and still damage the erythrocytes. It is more likely that some filterable product evolving from microsomal lipid peroxidation is involved. Continuing NADPH oxidation after filtration could not be responsible for the prelytic damage we observed (Fig. 2). NADPH oxidase activity in the active filtrate from the peroxidizing microsomal system was no more than 3 percent of the NADPH oxidase activity of the complete system before filtration. In two experiments, we found that, if the microsomal concentration of the complete system was diluted to 8 percent of its original NADPH oxidase activity, the capacity to produce prelytic damage to red cells disappeared; and in one experiment a dilution as high as 20 percent of original activity was also inert. Continuing liver microsomal lipid peroxidation after filtration also could not be responsible for the prelytic damage we observed (Fig. 2) for two reasons. (i) The capacity of filtrates from calcium microsomes to produce MDA in the presence of NADPH is no more than 3 percent of activity before filtration. (ii) Furthermore, addition of EDTA to the filtrates at a final concentration of 0.15 m*M* completely prevents microsomal lipid peroxidation in the complete system before filtration. During incubation of the filtrates with red cells and EDTA (Fig. 2) there was no further production of MDA.

Our data support the idea that some product of microsomal lipid peroxidation, arising at a circumscribed locus within the cell, can traverse finite distances and effect damage at other locations. However, the chemical nature of such a product is not yet known.

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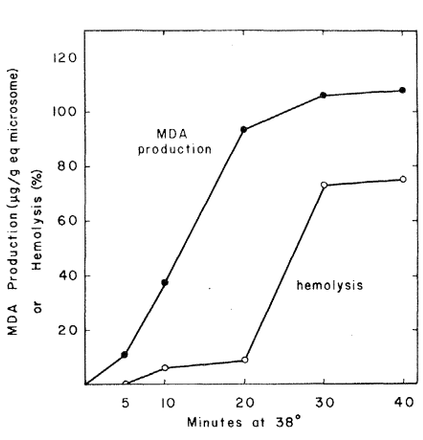


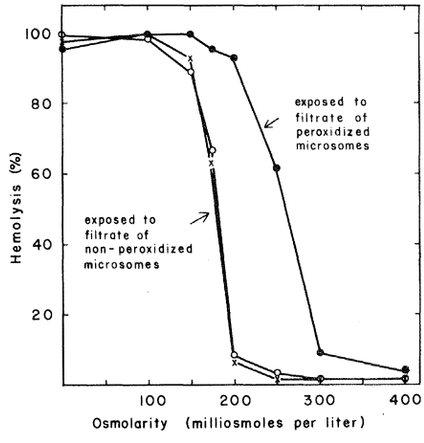


Fig. 1 (left). Lysis of erythrocytes preceded by malonic dialdehyde (*MDA*) production in a system of peroxidizing liver microsomes. Incubation was carried out aerobically at 38°C in a shaking water bath. The flask contained 0.5 percent normal rat erythrocytes (by volume), 25 mg-eq of microsomes per milliliter [a mg-eq of microsomes is the microsomal yield from 1 mg (wet weight) of liver], 0.3 m*M* NADPH, 0.05*M* sodium phosphate buffer, 0.09*M* sodium chloride, *p*H 6.6. All are expressed as final concentrations. MDA was measured by the thiobarbituric acid method (*16*) on portions that had been centrifuged to remove remaining erythrocytes and then treated with trichloroacetic acid to precipitate proteins and hemoglobin. Hemolysis was determined by measuring the optical density of the supernatant fraction from a portion that had been centrifuged to remove remaining erythrocytes, ghosts, and microsomes. The percent of hemolysis is 100 times the measured optical density at 542 nm divided by the optical density yielded by an equal concentration of erythrocytes hemolyzed in distilled water. Fig. 2 (right). Osmotic fragility induced in erythrocytes by an ultrafiltrate of a peroxidizing microsomal system. All flasks were initially incubated at 38°C for 10 minutes and then filtered under an atmosphere of 210 torr through an asbestos fiber filter (Gelman, A-E) layered on top of a 0.2-$\mu$m ultrafilter (Gelman, GA-8). At this point EDTA and erythrocytes were added to the filtrates, which were then incubated at 38°C for an additional 60 minutes. Erythrocytes were recovered and subjected to an osmotic fragility study modified from the procedure described by Cartwright (*17*). MDA was determined immediately after filtration. Flask 1 (•) contained NADPH (0.3 m*M*) plus 25 mg-eq of microsomes per milliliter incubated for 10 minutes in 0.09*M* NaCl buffered at *p*H 6.6 with 0.05*M* sodium phosphate (final volume, 36 ml) and then subjected to ultrafiltration; then 26.6 ml of the filtrate was brought to a final volume of 28 ml by the addition of EDTA (0.15 m*M*) and erythrocytes (0.5 percent). Incubation of this mixture was continued for 60 minutes. Flask 2 (○) contained a mixture similar to that of flask 1, except that NADPH was omitted. Flask 3 (×) was similar to flask 1 except that NADPH was added to the filtrate incubation mixture at the beginning of the 60-minute incubation period. All additions are expressed as final concentrations. Microsomes were prepared by aggregation in calcium media (*15*). MDA values were 1.24 and 0.5 $\mu$g per milliliter of incubation mixture for flasks 1 and 3, respectively.

**References and Notes**

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