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NADPH-DEPENDENT MICROSOMAL LIPID PEROXIDATION AND THE PROBLEM OF PATHOLOGICAL ACTION AT A DISTANCE

NEW DATA ON INDUCTION OF RED CELL DAMAGE*

MARK K. RODERS, ERIC A. GLENDE, JR. and RICHARD O. RECKNAGEL

Department of Physiology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

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Abstract—Lipid peroxidation occurs *in vitro* in a system consisting of rat liver microsomes and NADPH. If a small quantity of red cells is added, they will hemolyze. Lipid peroxidation (i.e. malonic dialdehyde evolution) always precedes red cell hemolysis in our system. This finding is contrary to earlier experiments of others, in which much higher concentrations of red cells were used, and in which certain other conditions varied from those used in our work. Addition of EDTA, which prevented lipid peroxidation, completely prevented red cell hemolysis. When aminopyrine was added, there was vigorous production of formaldehyde and neither lipid peroxidation nor red cell hemolysis occurred. Neither malonic dialdehyde nor hydrogen peroxide is responsible for the red cell hemolysis. If EDTA and erythrocytes are added to a microsomal system that has a prior history of NADPH-induced lipid peroxidation, the erythrocytes show prelytic damage, which can be detected by an osmotic fragility test. If erythrocytes are added to a system in which microsomal lipid peroxidation has been prevented (by the presence of EDTA), there is no evidence of osmotic fragility. These experiments suggest that some product or products arising in the peroxidizing microsomal lipids are capable of producing red cell damage.

Lipid peroxidation is a pathological condition with devastating consequences. It has been widely investigated because of its suspected role in a variety of disorders which often, although not always, stem from toxigenic sources. Lung damage, liver injury, and certain erythrocyte pathologies are among the types of conditions which have been most often studied. These disorders have been reviewed elsewhere [1–3]. Although lipid peroxidation produces structural and functional deformities, exact mechanisms are unknown. One possibility is that all the end stage pathological manifestations stem from the actual physical destruction of the lipoprotein membrane. This is the so called "hole in the membrane hypothesis" [4]. This would explain, for example, the loss of soluble enzymes into the plasma as a consequence of carbon tetrachloride-induced liver cell injury. Another possibility is that toxic products are produced during the course of lipid peroxidation. These toxic products would arise at a localized site within the cell and have the capacity of inducing pathological effects at distant loci. This communication is concerned with the second of these two ideas.

It has been known for 13 years that when liver microsomes are incubated with NADPH, and without any electron accepting terminal substrate, lipid peroxidation will occur [5]. Subsequently it was reported that if erythrocytes are present in this peroxidizing microsomal system they will hemolyze [6]. Since lipid peroxidation is associated with a variety of pathological conditions, it seems reasonable to suspect that the erythrocytic hemolysis is due to some toxic product arising from the microsomes as their lipids undergo peroxidation. Supportive of this idea is the finding that erythrocytes hemolyze when incubated with linoleic hydroperoxide [7, 8]. However, in a more recent study it was concluded that the red cell hemolysis was not due to a product of peroxidized microsomal lipids. Rather, a free radical hypothesis was suggested [9]. According to the hypothesis (Fig. 1), hydroxyl radical generated by the microsomal mixed function oxidase system attacks the erythrocytes. The experiments reported in this communication do not disprove the free radical hypothesis. However, they do lend highly significant support to the view (Fig. 1) that a toxic product, arising from peroxidizing microsomal lipids, is involved in the hemolysis.

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MATERIALS AND METHODS

Animals. Male rats of the Sprague Dawley strain from Zivic–Miller or Flow Labs were maintained on Purina Lab chow and water *ad lib.* The rats used in these experiments were generally between 150 and 300 g.

Chemicals. NADPH and catalase, were obtained from the Sigma Chemical Co., 1,1,3,3-tetraethoxy-propane and 2-thiobarbituric acid from Eastman Organic Chemicals, aminopyrine from Matheson Coleman & Bell, disodium (ethylenedinitrilo) tetra-acetate (EDTA) from the J. T. Baker Chemical Co., and heparin sodium from The Upjohn Co. All other chemicals were of reagent grade purity.

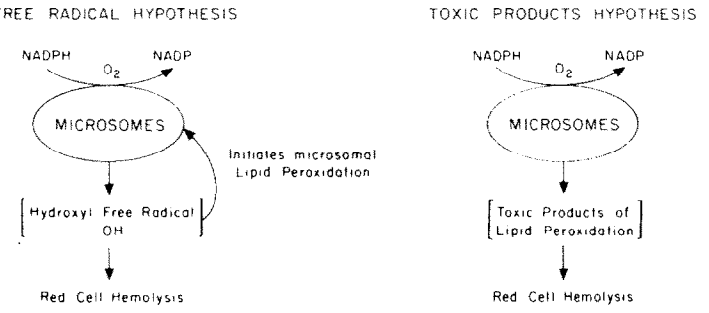

Fig. 1. Free radical hypothesis and toxic products hypothesis for red cell hemolysis in a NADPH–microsome system *in vitro*.

Preparation of microsomes. Rats were decapitated and allowed to exsanguinate. The livers were quickly excised, washed in 0.154 M NaCl, and the wet weight was determined. A 10% (w/v) whole liver homogenate was prepared in 0.09 M NaCl buffered with 0.05 M sodium phosphate, pH 6.6. This homogenate was centrifuged at 2700 *g* for 10 min in the SS-34 rotor of the Sorvall RC2-B centrifuge. The post-mitochondrial supernatant fraction was centrifuged at 100,000 *g* for 30 min in a Beckman preparative ultracentrifuge using a type 50.1 rotor. The resulting microsomal pellet was rehomogenized and diluted in the above buffer so that the microsomal yield from each gram of liver was contained in 10.0 ml buffer. This yields a suspension with a concentration of 100 mg eq microsomes/ml, where a mg eq microsomes is defined as the microsomal yield from 1.0 mg wet weight of liver. All procedures involved in the isolation of these microsomes were conducted at 0–4°.

Preparation of erythrocytes. Blood was taken from the aorta using heparin as an anticoagulant. The blood was centrifuged at 1100 *g* for 5 min in a table model International Clinical centrifuge using a type 809 rotor. The supernatant fraction and buffy coat were discarded. The packed red cells were gently resuspended and washed three times in 0.9% NaCl resedimenting as above between washes. The final sedimentation was at 1100 *g* for 15 min. The volume of the packed red cells was then determined, and they were diluted 1:5 with 0.9% NaCl to yield a 20% (v/v) erythrocyte suspension. A 1% (v/v) erythrocyte suspension is defined as 1.0 ml of packed erythrocytes/100.0 ml of suspension. The erythrocytes were stored for one night at 4° before being used.

Preparation of malonic dialdehyde (MDA). Malonic dialdehyde was prepared by acid hydrolysis of 1,1,3,3-tetraethoxypropane according to the method of Kwon and Watts [10]. The yield of MDA was determined by using a molar extinction coefficient at 267 nm of 3.18×10^4. To test for a possible direct effect on red cells, the hydrolysate containing MDA was adjusted to pH 6.6 and added to erythrocyte suspensions.

Determination of malonic dialdehyde. An aliquot from the reaction flask was centrifuged at 1500 *g* for 3 min to sediment any remaining red cells and ghosts. The supernatant fraction was acidified with trichloroacetic acid (5% acid, w/v, final concentration) and centrifuged to remove the denatured microsomes and hemoglobin. MDA content of the final clear supernatant fraction was determined according to Recknagel and Ghoshal [11].

Determination of hemolysis. An aliquot from the reaction flask was centrifuged at 1500 *g* for 3 min to sediment remaining erythrocytes and ghosts. To remove the remaining microsomes the supernatant fraction was further centrifuged at 20,000 *g* for 60 min in the SS-34 rotor of the Sorvall RC2-B centrifuge. The optical density of the final supernatant fraction was determined at 542 nm. The percentage of hemolysis was calculated by comparison with the optical density yielded by an equal concentration of erythrocytes completely hemolyzed in distilled water.

Aminopyrine demethylase activity. Aminopyrine demethylase was determined by measuring the amount of formaldehyde produced by microsomes in the presence of aminopyrine. Formaldehyde was determined according to the method of Nash [12].

Catalase assay. Determination of the enzymic activity of purchased catalase was made according to the procedure described in the Sigma Chemical Co. catalogue (1974). Our only deviation from the procedure described therein is that we assayed at pH 6.6 rather than 7.0. This was done in order to determine how active the catalase would be in a system of peroxidizing microsomes at pH 6.6. The additions of catalase described in this paper are based on the following definition: 1 unit catalase will decompose 1 μmole H_2O_2/min at pH 6.6 at 25°, while the H_2O_2 concentration falls from 10.3 to 9.2 μmoles/ml of reaction mix.

Osmotic fragility test. The osmotic fragility of erythrocytes was determined as follows: 2.0 ml aliquots from reaction mixtures containing erythrocytes were placed in a series of eight round bottom 13 × 100 mm culture tubes. The tubes were centrifuged at 1100 *g* for 5 min in a table model International Clinical centrifuge equipped with a type 809 rotor. The supernatant fraction was removed by delicate vacuum aspiration. The erythrocyte pellets were gently resuspended in 5.0 ml from one of a graded series of NaCl solutions which were 0–400 mOsM. The tubes were then allowed to stand at ambient room temperature for 30 min after which they were centrifuged at 1100 *g* for 10 min. The optical density of the supernatant fraction was determined at 542 nm. To convert optical density to percent hemolysis the optical density at any one given osmolar concentration of NaCl was divided by the optical density of the tube representing

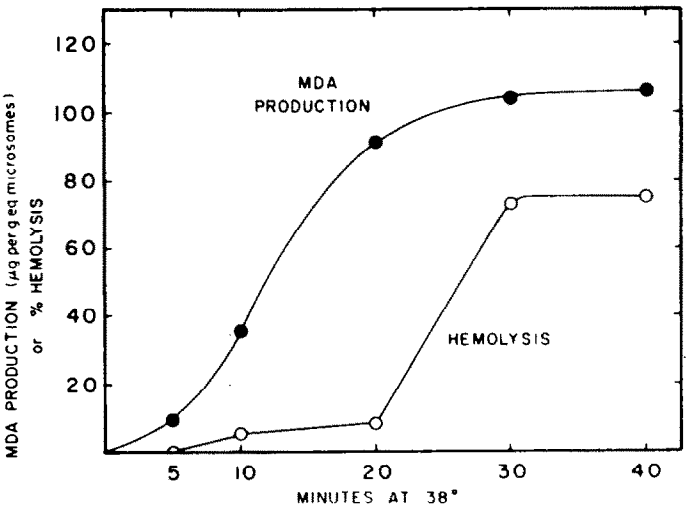

Fig. 2. Malonic dialdehyde production and hemolysis in a system of rat liver microsomes, erythrocytes and NADPH. Incubation was conducted aerobically at 38° using a water bath with agitation. MDA and hemolysis were determined at various times on aliquots from the same flask which contained: 25 mg eq microsomes/ml, 0.3 mM NADPH, 0.5% (v/v) erythrocytes, 0.09 M NaCl, and 0.05 M sodium phosphate, pH 6.6. The final volume of flask contents was 40.0 ml.

complete hemolysis of red cells. The resulting value was multiplied by 100.

Incubation conditions. Incubations were carried out at 38°, in a water bath with agitation. The gas phase was air. A typical reaction system contained 25 mg eq microsomes/ml, 0.5% (v/v) erythrocytes, 0.3 mM NADPH, 0.09 M NaCl, and 0.05 M sodium phosphate, pH 6.6. All additions are expressed as final concentrations. Final reaction flask volumes ranged between 6.0 and 50.0 ml. In all cases the incubation media contained 300 osmoles/l. in order to assure isotonicity with the erythrocytes. For exact experimental conditions for particular experiments, see the appropriate figure legends.

RESULTS

Time course of microsomal lipid peroxidation and erythrocytic hemolysis. When a system containing rat liver microsomes, NADPH and erythrocytes was incubated, MDA appeared well before the red cells lysed (Fig. 2). Since the evolution of MDA indicates that lipid peroxidation has occurred, this sequence of events is consistent with the idea that some product from the peroxidized lipids is responsible for the subsequently occurring hemolysis. Previous workers had reported that hemolysis preceded MDA appearance [9]. However, we have consistently obtained opposite results. In our experiments, MDA evolution always precedes hemolysis.

Experiments involving addition of malonic dialdehyde. Yields of MDA found in the microsome–NADPH–erythrocyte system, after 60 min of incubation, are usually about 100 μg MDA/eq g of liver microsomes. When this is recalculated on a molar basis, the MDA yield is seen to be 3.5×10^{-5} M. Hemolysis occurs in systems where this amount of MDA is produced; however, when MDA is prepared from 1,1,3,3-tetraethoxypropane and incubated with erythrocytes at final concentrations ranging from 2.0×10^{-5} M to 3.6×10^{-3} M, no hemolysis occurs. Thus, MDA added at levels 100 times in excess of what is generally observed produced no hemolysis.

Inhibition of microsomal lipid peroxidation and prevention of hemolysis with EDTA. When microsomes, NADPH and red cells are incubated in the presence of added EDTA, neither microsomal lipid peroxidation nor erythrocytic hemolysis occurs (Table 1, line 2). It has previously been shown that EDTA does not inhibit microsomal mixed function oxidase activity [13–16]. The results (Table 1) clearly indicate that hemolysis does not take place when lipid peroxidation is prevented by addition of EDTA, omission of NADPH, or absence of microsomes. In all of our experiments using the microsome–NADPH–erythrocyte system, hemolysis occurred only when microsomal lipid peroxidation had taken place.

Use of aminopyrine to prevent hemolysis during active microsomal electron transport. When NADPH is oxidized by microsomes in the presence of aminopyrine, lipid peroxidation does not occur. Note that no hemolysis takes place if red cells are added to this system (Fig. 3). Although active electron transport is occurring, as evidenced by the production of formaldehyde, the absence of hemolysis clearly indicates that mixed function oxidase activity *per se* is insufficient to account for the red cell damage. In suitable control systems (data not shown) in which aminopyrine was not added, there was vigorous production of MDA followed by up to 80 per cent hemolysis during 60 min of incubation.

Addition of catalase to the microsome-NADPH-erythrocyte system. Since hydrogen peroxide is a known hemolytic agent [17, 18], which has been detected in microsomal systems during NADPH oxidation [19–21], we considered the possibility that it could be the entity responsible for the red cell lysis. Addition of catalase did not depress MDA production nor did it protect against hemolysis (Table 2). Based on the data of Thurman *et al.* [20], we calcu-

Table 1. Effect of added EDTA on lipid peroxidation and hemolysis*

Line No.	Contents	MDA production†	% Hemolysis
1	Microsomes, NADPH, erythrocytes	74.7	90
2	Microsomes, NADPH, erythrocytes, EDTA	1.1	3
3	Microsomes, erythrocytes	4.9	3
4	NADPH, erythrocytes	0.4	6
5	Erythrocytes in isotonic buffer alone		5
6	Erythrocytes in distilled H_2O		100

* Final concentration of the indicated flask contents was 25 mg eq microsomes/ml, 0.3 mM NADPH, 0.5% (v/v) erythrocytes and 0.1 mM EDTA. Incubation media was 0.09 M NaCl and 0.05 M sodium phosphate, pH 6.6. Final volume was 16.0 ml. Incubation was for 60 min at 38°.

† Expressed as μg MDA/eq g of liver microsomes.

lated that the microsome–NADPH–erythrocyte system that we used could produce hydrogen peroxide at a maximal rate of 9.0×10^{-9} moles/min/ml; however, addition of catalase at a concentration of 50,000 units/ml (Table 2) would decompose hydrogen peroxide at a theoretical rate of 5.0×10^{-2} moles/min/ml. In addition, where hydrogen peroxide has been reported as a hemolytic agent [17], the concentration used (0.8 M) was more than 1000 times greater than the level we calculate could possibly be present in the microsome–NADPH–erythrocyte system. Thus, calculations based on the work of others, as well as our own experimental results (Table 2), make it extremely unlikely that hydrogen peroxide is the hemolytic agent.

Time of peak hemolytic activity in a peroxidizing microsome–NADPH system. A microsome–NADPH system was incubated at 38°. At various times aliquots were removed and added to suspensions of erythrocytes. Without significant time delay, each of the combined systems containing peroxidizing microsomes and red cells was then incubated for an additional 40 min, after which the extent of hemolysis was determined. Maximum hemolysis occurred if the peroxidizing microsomal system was brought into contact with the erythrocytes 10 min after the enzyme reaction was started by the addition of NADPH (Fig. 4). By 30 min most of the hemolytic activity had disappeared. The experimental result shown in Fig. 4 confirms an essentially identical finding of Pfeifer and McCay [9]. The significance of this result is that it indicated that, for our conditions, maximum hemolytic activity is present after 10 min of incubation. MDA evolution was also monitored. It may be noted that peak hemolytic activity occurred at the same time (10 min) as the maximum rate of evolution of MDA.

Survival of anti-red cell activity in a system with a prior history of microsomal lipid peroxidation. Microsomes were prepared and distributed into three flasks. NADPH was added to the first flask and lipid peroxidation was allowed to take place for 10 min. EDTA and erythrocytes were then added. After a further 60-min incubation, the red cells exhibited osmotic fragility (Fig. 5). The second flask was a basic control; NADPH was never present, there was no lipid peroxidation, and the red cells exhibited normal osmotic properties. In the third flask, NADPH was present during the initial 10 min of incubation; however, microsomal lipid peroxidation was prevented by the addition of EDTA. When red cells were then added and further incubated, they showed no evidence of osmotic fragility.

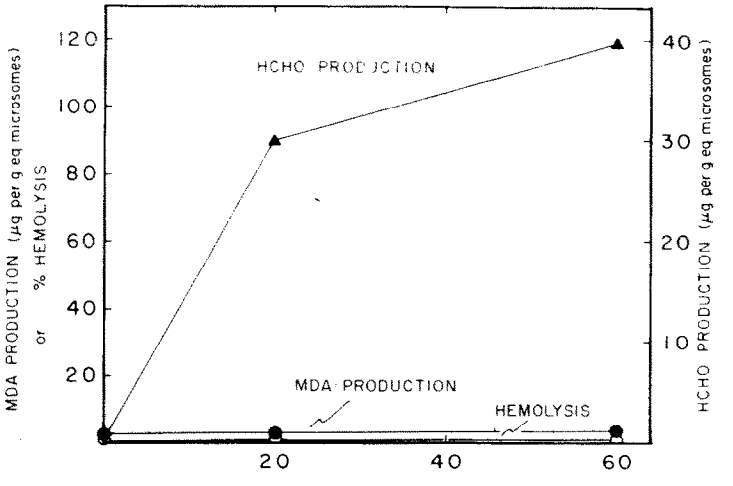

Fig. 3. Formaldehyde and malonic dialdehyde production, and hemolysis in a system of rat liver microsomes, NADPH, erythrocytes and aminopyrine. MDA, HCHO and hemolysis were determined at various times on aliquots from the same flask. Conditions are as in Fig. 2 except that 10.0 mM aminopyrine was present. The final volume of flask contents was 20.0 ml. Abscissa: min at 38°.

Table 2. Effect of catalase on a system of peroxidizing microsomes and erythrocytes*

Flask No.	Contents	MDA production†	% Hemolysis
1	Microsomes, NADPH, erythrocytes	99.9	75
2	Flask 1 + 50 units catalase/ml	102.4	77
3	Flask 1 + 500 units catalase/ml	101.9	82
4	Flask 1 + 5,000 units catalase/ml	103.0	83
5	Flask 1 + 50,000 units catalase/ml	102.9	77
6	Erythrocytes in distilled H_2O		100

* Conditions are as in Fig. 1, except that catalase was added at various concentrations where indicated. Final volume of flask contents was 6.0 ml.

† Expressed as μg MDA/eq g of liver microsomes.

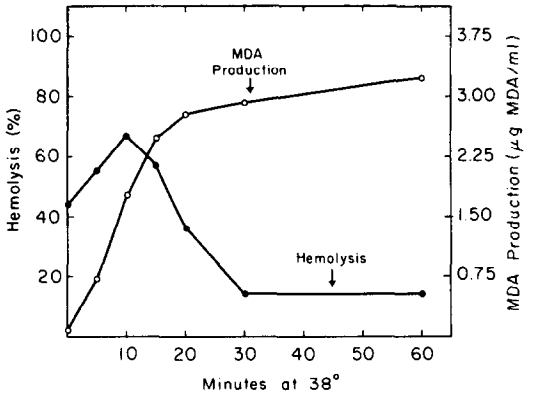

Fig. 4. Red cell hemolysis and malonic dialdehyde evolution in a peroxidizing microsome–NADPH system. A flask containing 25 mg eq liver microsomes/ml in 0.09 M NaCl and 0.05 M sodium phosphate buffer, pH 6.6, was pre-incubated for 5 min at 38° to assure temperature equilibration. The phosphate–NaCl medium supports vigorous lipid peroxidation and it is 300 mOsM, i.e. isotonic with respect to red cells. Lipid peroxidation was then induced at time = 0 by adding NADPH (0.3 mM final concn). At various times thereafter, 3.0 ml aliquots of the peroxidizing microsomal mixture were added to tubes containing erythrocytes (final concentration of erythrocytes was 0.5%, v/v). Each tube containing microsomes and erythrocytes was then further incubated at 38° for 40 min. The extent of hemolysis in each tube was then determined. The MDA determinations refer to the amount of MDA present in the initial incubation flask at the time indicated.

DISCUSSION

Lipid peroxidation occurs readily when liver microsomes oxidize NADPH in the absence of exogenous terminal substrates. As a result of this peroxidation, microsomal enzymes such as glucose 6-phosphatase, cytochrome P-450, and amino pyrine demethylase are destroyed [16, 22, 23]. When microsomal lipid peroxidation takes place in the liver *in vivo*, such phenomena as mitochondrial destruction, loss of soluble intracellular enzymes into the plasma, lysosomal disruption, and nuclear damage are known to occur [2, 23]. From a mechanistic point of view, a key problem is to account for the widespread nature of these structural and functional derangements. In other words, if lipid peroxidation occurs initially at one circumscribed location within the cell, e.g. at the cytochrome P-450 locus of the endoplasmic reticulum, what are the significant events which eventually result in destructive manifestations at other locations? The NADPH–microsome–red cell system provides an experimental model for the study of this problem.

To account for the hemolysis that was observed in a microsome–NADPH–erythrocyte system, Pfeifer and McCay [9] suggested a free radical hypothesis (Fig. 1). According to this hypothesis, hydroxyl radical is generated by the microsomal electron transport system and it directly attacks the red cells. Hydroxyl radical was also postulated to be the initiator of microsomal lipid peroxidation. We accept the idea that hydroxyl radical may be responsible for initiating microsomal lipid peroxidation. The role of free radicals in the autoxidation of lipids is generally recognized. The recent report that hydroxyl radical has been detected in microsomal systems by the use of spin trapping confirms earlier indications of its presence [24]. Given the typical conditions employed *in*

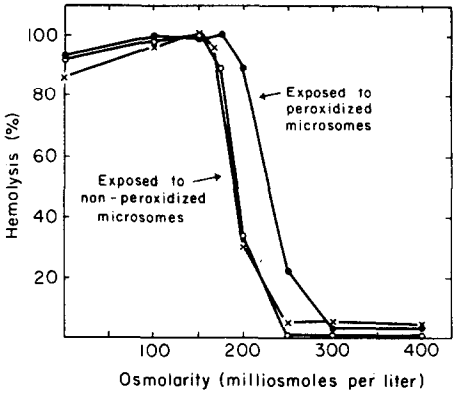

Fig. 5. Effect of the time when EDTA is added on osmotic fragility produced by microsomes. Three flasks containing 25 mg eq microsomes/ml, 0.09 M NaCl and 0.05 M sodium phosphate, pH 6.6, were pre-incubated at 38° for 5 min to assure temperature equilibration. The following additions were made at the indicated times. Flask 1 (●) 0 min: 0.3 mM NADPH; 10 min: 0.15 mM EDTA and 0.5% (v/v) erythrocytes. Flask 2 (○) 0 min: no additions; 10 min: 0.15 mM EDTA and 0.5% (v/v) erythrocytes. Flask 3 (×) 0 min: 0.15 mM EDTA and 0.3 mM NADPH; 10 min: 0.5% (v/v) erythrocytes. After addition of erythrocytes, all flasks were further incubated at 38° for 60 min. An osmotic fragility study was then conducted as described in Materials and Methods. All additions are expressed as final concentrations. MDA calues after the initial 10-min incubations were 1.69 μg MDA/ml in flask 1 and 0.09 μg MDA/ml in flask 3.

vitro, viz. isolated microsomes plus NADPH, we believe that it is not all unlikely that a radical, such as hydroxyl radical, could be responsible for the initiation of microsomal lipid peroxidation in such systems. The environment of the endoplasmic reticulum has been drastically altered. Other sub-cellular organelles and cytosol have been removed, thereby depriving the microsomes of water soluble antioxidants. During the incubations *in vitro*, electron pressure is being supplied by reducing equivalents as NADPH, and the system is under relatively high oxygen tension. This system is biochemically meaningful and useful. However, it is physiologically quite different from what is present in the cell. In this very refined environment *in vitro*, NADPH is readily oxidized. Hydroxyl radical most likely arises at some localized site within the endoplasmic reticulum, e.g. cytochrome P-450. In the presence of terminal substrates, hydroxylation reactions will occur and the hydroxyl radical will thus be constrained along normal metabolic pathways. In the absence of substrates, it is plausible to envision hydroxyl radical as "leaking out" of its normally meaningful sphere of biological influence into the surrounding lipoidal matrix. When lipid soluble antioxigenic potential (e.g. vitamin E) is critically depleted, lipid peroxidation will ensue. We believe that this part of the free radical hypothesis (Fig. 1) is cogent and reasonable. However, in our opinion, that aspect of the hypothesis postulating direct radical-induced hemolysis contains a serious theoretical weakness. Hydroxyl radical is a highly reactive, extremely unstable species. It undergoes reactions nearly at the rate at which it collides with other molecules [25]. The chemistry of hydroxyl radical argues against the possibility that it could survive long enough to attack the erythrocytes. It is the high reactivity of hydroxyl radical which makes it a likely initiator of microsomal lipid peroxidation. This same high reactivity makes it less likely that it is the direct hemolytic agent.

The significance of the experiments that we have described is that they support an alternate interpretation for the hemolysis observed in the microsome–NADPH–erythrocyte system. The toxic products hypothesis (Fig. 1) maintains that some toxic entity is arising in the microsomal lipids as they undergo peroxidative decomposition. According to the hypothesis, the toxic product or products are responsible for inducing the erythrocytic hemolysis. The fact that lipid peroxidation always occurs before hemolysis takes place (Fig. 2) contradicted earlier work [9] and supported a toxic products hypothesis. With regard to why our findings are in disagreement with those of previous workers, we can only offer two possibilities. First, we used much more dilute concentrations of erythrocytes (0.5 vs 10%). We found (unpublished) that when a 10% (v/v) red cell concentration is used the fraction of cells actually hemolyzed is very small (approximately 5 per cent hemolysis). Second, we monitored both MDA production and hemolysis from a single incubation flask which contained both microsomes and erythrocytes (see Materials and Methods). The earlier workers had monitored MDA production from a flask which contained only microsomes. They determined the amount of hemolysis from a separate flask which contained both red cells and microsomes. We feel that our system which employs far fewer red cells, and in which we simultaneously monitor MDA production and extent of hemolysis from the same reaction flask, correctly defines the time relationship between these two processes. The significance of this experiment (Fig. 2) is that it suggested to us that microsomal lipid peroxidation might be the key to the hemolysis. The other experiments we described strengthened this view. For example, when EDTA was added to the system (Table 2), there was no lipid peroxidation and no hemolysis. With regard to this experiment, the following comment may be made. If the hemolytic agent is a free radical coming from the microsomal electron transport system, and if the mechanism of red cell hemolysis, after a free radical attack, involves peroxidation of lipids in the erythrocyte membrane, then the capacity of EDTA to inhibit lipid peroxidation may be protecting the red cell membrane directly, thereby preventing the hemolysis from occurring. In other words, one might argue that, even if the hemolytic agent is a free radical, the EDTA could prevent the hemolysis by stopping oxidation at the locus of the erythrocyte plasmalemma. However, the experiment described in Fig. 5 shows that red cells can be damaged in the presence of EDTA, when placed in contact with a toxic entity that has been produced by peroxidizing microsomes. This observation strongly augments the significance of the EDTA experiment recorded in Table 1.

The fact that anti-red cell activity, expressed as osmotic fragility, persists in a system which has a history of microsomal lipid peroxidation (Fig. 5) indicates that some product or products evolved in the peroxidized microsomes could be responsible for the red cell damage. The ability to produce prelytic damage was present only in the flask which had undergone peroxidation. The capacity to damage erythrocytes could not be due to continuing microsomal lipid peroxidation after addition of EDTA. The concentration of EDTA used (0.15 mM) completely prevents any further lipid peroxidation, as evidenced by lack of any additional MDA evolution. Regarding the aminopyrine experiment, the critical observation was that there was no hemolysis even though active electron transport was occurring over the microsomal electron transport system. The fact that hemolysis did not occur, even though microsomal electron transport was occurring, offers no support for the free radical hypothesis. On the other hand, the finding that absence of hemolysis correlated with absence of lipid peroxidation is consistent with the toxic products hypothesis.

The experiments presented in this communication do not disprove the hypothesis that hydroxyl radicals, arising from microsomal oxidation of NADPH, may be involved in the red cell hemolysis observed when red cells are present in systems of peroxidizing liver microsomes. Neither do the experiments presented here disprove that radical mechanisms may be involved at the level of the red cells when either lysis or prelytic damage occurs. The significance of the experiments reported here is that they clearly indicate that the red cell membrane can be subjected to prelytic damage by some product or products arising in the microsomal membrane, as its constituent lipids

undergo peroxidative decomposition. The fact that microsomal lipid peroxidation can produce prelytic damage in red cells suggests the possibility that the frank hemolysis observed when the red cells are present in mixtures of peroxidizing microsomes may be due, at least in part, to the action of products arising from degenerative alterations in microsomal membranes as a result of lipid peroxidation.

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