Changes in Fatty Acid Metabolism after Erythrocyte Peroxidation: Stimulation of a Membrane Repair Process

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Research Article

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The lipids of the membranes of erythrocytes were labeled with saturated and unsaturated fatty acid. When these erythrocytes were subsequently incubated with hydrogen peroxide, both types of fatty acid were transferred from superficial erythrocyte neutral lipids into phosphatidylethanolamine. However, the unsaturated fatty acids of phosphatidylethanolamine were subsequently altered by hydrogen peroxide, whereas the saturated fatty acids were not. The cumulative effect of these processes was a relative decrease in unsaturated fatty acid and an increase in saturated fatty acid in the phosphatidylethanolamine of the erythrocyte membrane.

The net effect of these events represents the operation of repair processes which distort the usual fatty acid composition of erythrocyte membranes in the presence of H_2O_2. This distortion may contribute to membrane permeability changes which occur during peroxide exposure and which precede the eventual hemolysis of these cells.

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Changes in Fatty Acid Metabolism after Erythrocyte

Peroxidation: Stimulation of a Membrane Repair Process

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INTRODUCTION

Lipid peroxidation occurs when unsaturated fatty acids are exposed to a free radical generating system such as hydrogen peroxide (1). The presence of vitamin E or a similar antioxidant will prevent this process (2).

Prelytic alterations in lipids have been reported in vitamin E–deficient red blood cells during incubation in the presence of a H_2O_2-generating system. In the vitamin E–deficient rat, Jacob and Lux have shown that membrane phosphatidylethanolamine (PE)[1] is significantly decreased during such incubation (3). In man, erythrocytes from patients with abetalipoproteinemia exhibited a prelytic loss of PE, phosphatidylserine (PS), and polyunsaturated fatty acids during exposure to H_2O_2 (4). Also in man, vitamin E-deficient erythrocytes from newborn infants exhibited lipid peroxidation as measured by the thiobarbituric acid reaction after incubation with H_2O_2 (5).

PE contains a high concentration of polyunsaturated fatty acids (6) and hence may be especially sensitive to lipid peroxidation. It has been shown that a significant renewal pathway for PE fatty acid involves the transfer of fatty acid from a superficial neutral lipid pool through phosphatidylcholine (PC) into PE (7). Unlike red blood cell (RBC) PC, RBC PE is not partially sustained by passive exchange with plasma PE since the latter is virtually undetectable (8).

The purpose of the studies reported here was to examine the renewal pathways for RBC PE when vitamin

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[1] *Abbreviations used in this paper:* FA, fatty acid; KHB, Krebs-Henseleit buffer; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RBC, red blood cell.

E–deficient red blood cells were incubated with H_2O_2. The results demonstrated an increase in the incorporation of fatty acid into RBC PE, which was consistent with an H_2O_2-stimulated membrane "repair process." However, saturated fatty acid accumulated in PE to a much greater extent than unsaturated fatty acid. This remodeling of membrane fatty acid composition may contribute to membrane stiffness and permeability changes and hence to the rheologic abnormalities of peroxide-injured cells.

METHODS

1. Materials

Crystallized human serum albumin for the incorporation of labeled fatty acids was obtained from Dade Reagents Inc., American Hospital Supply Corp., Evanston, Ill. Linoleic acid-1-^{14}C, palmitic acid-9,10-^{3}H, and arachidonic acid-1-^{14}C were obtained from Applied Science Laboratories Inc., State College, Pa. Defatted albumin for the removal of superficial red cell fatty acid was prepared from human fraction V supplied by the Massachusetts State Department of Health by equilibration with activitated charcoal as described by Chen (9). H_2O_2, as Superoxol, was obtained from Fisher Scientific Company, Pittsburgh, Pa.

2. Cell collection and preparation

Heparinized peripheral venous blood was obtained from children with clinical histories compatible with vitamin E deficiency (10–12). Samples exhibiting greater than 80% hemolysis in the peroxide hemolysis test determined by the method of Rose and Gyorgy were used for further studies (13). The red cells were separated from the serum and residual leukocytes and platelets with multiple washes in Krebs-Henseleit buffer (KHB) at pH 7.4 as previously described (14). When reconstituted to an hematocrit of 6%, the white blood cell count was less than 100 cells/ml^3.

3. Lipid analysis

Erythrocyte lipids were extracted and separated according to methods previously published (14). Major lipid components were isolated by thin-layer chromatography on Silica Gel H plates obtained from Brinkman Instruments Inc., Westbury, N. Y. Appropriate spots were identified by iodine staining. After complete evaporation of the iodine, they were scraped from the plate, and determinations of lipid phosphorous and radioactivity were made on fractions of sonicated suspensions of this material by the direct methods of Parker and Peterson (15) and Snyder and Stephens (16). Methyl esters were prepared using boron trifluoride (17). Gas-liquid chromatography was performed by methods similar to those of Dodge and Phillips (6) using an 8 ft glass column packed with 7% EGSS-X on Gas-Chrom P, 100–120 mesh, with a hydrogen flame ionization detector. The carrier gas flow rate was 30 ml/min, and the column temperature was maintained at 190°C and the injector at 215°C. Retention times were determined daily by comparison with a known standard mixture of fatty acid methyl esters. Peak areas were calculated by triangulation and were corrected in proportion to the molecular weights of the component fatty acids. Butylated hydroxytoluene which was usually added as an antioxidant emerged from the column with a

retention time similar to that of methyl myristate, and thus myristic acid was not independently determined. The gas-liquid chromatography methods used here were not capable of distinguishing the geometric isomers of unsaturated fatty acids or of separately resolving dimethylacetal derivatives of plasmalogens (6).

4. Radioactivity determinations

Radioactivity measurements were made in a Packard Tri Carb, model 3370 scintillation spectrometer. Buhler's solution was used as a scintillating fluid (18). ^{3}H and ^{14}C radioactivities were separated as previously described (14). Quench corrections were made for each sample by the use of individual internal standards. Radioactive fatty acids were purified by thin-layer chromatography (19) before being complexed with defatted albumin (14).

5. Experimental procedures

A. Incorporation of radioactive fatty acid into red cell phosphatides. Washed red cells (4% hematocrit in KHB) were incubated at 37°C in the presence of radioactive fatty acids bound to albumin (final concentration of albumin 300 mg/100 ml [14], and final specific activity of fatty acid albumin complexes: arachidonic acid-^{14}C, 17 mCi/mmole, and palmitic acid-^{3}H, 27 mCi/mmole) with an equal volume of KHB with or without 2% w/v H_2O_2. The reaction was terminated by three washes at 4°C with KHB containing 5% of a solution saturated with thymol (5). The lipids of the cells were then extracted, separated, and analyzed as in Methods sections 3 and 4.

B. Transfer of fatty acids into phosphatidylethanolamine. Red cells were prepared and incubated with saturated and unsaturated fatty acids without H_2O_2 as above. After a 2 hr incubation, the cells were washed free of unincorporated fatty acid with KHB and reincubated at a final hematocrit of 4% with an equal volume of 2% H_2O_2 in KHB or with an equal volume of KHB. At each time period thereafter, KHB-containing thymol was added, the cells were centrifuged, the supernate was discarded, and the cell lipids were extracted and analyzed as above.

C. Transfer of fatty acid into phosphatidylethanolamine after removal of superficial membrane lipid pool. To assess the role of the neutral lipid in the repair process, cells were labeled without the presence of H_2O_2 as in Methods section 5 A using ^{3}H-labeled palmitic acid. Palmitic acid was used in these studies because, being fully saturated, it was unlikely that it would undergo peroxidative degeneration. The radioactivity labeled fatty acid in the superficial fatty acid (FA) pool was then replaced with nonradioactive fatty acid as follows: the media were removed by centrifugation, and the cells were washed twice with cold isotonic NaCl. The cells were then washed twice with a cold 1% solution of defatted human serum albumin in isotonic NaCl. The cells were then reincubated with 10 vol of autologous serum to reestablish a nonradioactive superficial membrane neutral lipid pool (14). The cells were finally washed in fresh isotonic NaCl and reincubated as in Methods section 5 B.

RESULTS

Quantitative changes in red blood cell phospholipids after incubation in hydrogen peroxide for 1 hr. A slight (6–16%) loss of total phospholipid before lysis
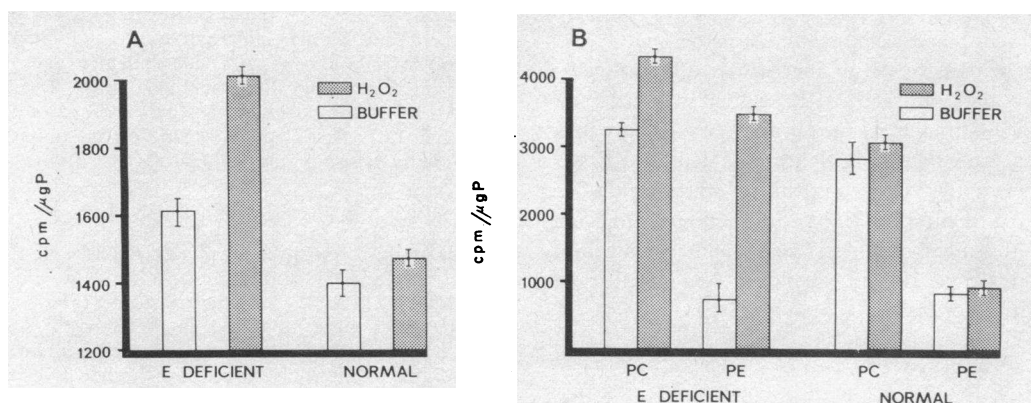
FIGURE 1 Influence of hydrogen peroxide on the incorporation of fatty acid into normal and vitamin E–deficient red blood cells. The cells were incubated with linoleic-1-^{14}C fatty acid for 1 hr, extracted, and counted. The results are expressed as counts per minute/microgram lipid phosphorous. Fig. 1 A represents the total radioactivity incorporated. Fig. 1 B represents the radioactivity incorporated into phosphatidylcholine and phosphatidylethanolamine. Incubation in buffer is represented by the clear bars, incubation in hydrogen peroxide by the cross-hatched bars. The points in this figure and in all subsequent figures represent average of duplicates. The ranges are indicated by brackets. See Methods section 5 A.

occurred in half of the samples of vitamin E–deficient red cells which were exposed to 1% H_2O_2 (Table I). When present, this loss was predominantly found in PE. PC, PS, phosphatidylinositol, and sphingomyelin were virtually unaffected before lysis. In contrast, the PE of normal red blood cells was unaffected during incubation in H_2O_2 (data not shown).

Influence of H_2O_2 on incorporation of fatty acids into red cell phospholipids. A representative study of the incorporation of linoleic acid into normal and vitamin
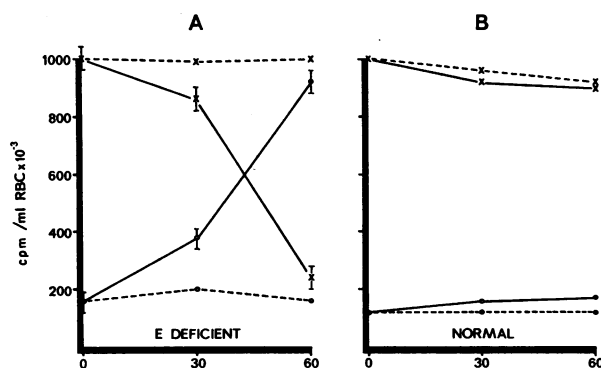

FIGURE 2 Changes in neutral lipid and PE fatty acid after incubation in hydrogen peroxide. Red cells were preincubated with palmitic-1-^{14}C fatty acid, washed with saline, and reincubated with hydrogen peroxide. The red cell lipids were extracted, separated by thin-layer chromatography and counted. The radioactivity was expressed as counts per minute/milliliter RBC. Quantitative changes in PE did not occur before lysis in this study. X = counts in neutral lipid; O = counts in PE. Dotted lines represent incubation in buffer, the solid lines represent incubation in H_2O_2. See Methods section 5 B.

E–deficient cells after a 1 hr incubation period is seen in Fig. 1 A. Incubation in buffer alone resulted in a greater total incorporation of fatty acid into the vitamin E–deficient cell than into the normal cell. The addition of H_2O_2 to the buffer further stimulated the incorporation of fatty acid into the E-deficient cell but had no effect on the normal cell.

The distribution of incorporated fatty acid is seen in Fig. 1 B. The specific activity of PE was increased by H_2O_2 in the E-deficient cell but was unaffected in the normal cell. In fact most of the increase in total incorporation of fatty acid due to H_2O_2 (Fig. 1 A) was accounted for by the increase in specific activity of phosphatidylethanolamine shown in Fig. 1 B.

Transfer of fatty acid into PE. The transfer of palmitic acid-^{3}H from neutral lipid into PE (Methods section 5 B) is seen in Fig. 2 A and 2 B. The distribution of radioactivity in the individual lipid classes was determined at 0, 30, and 60 min. In the E-deficient cell (Fig. 2 A), fatty acid present in neutral lipid was transferred into PE during incubation with H_2O_2 but not with buffer alone. The total quantity of PE remained stable during this 60 min interval. In normal cells (Fig. 2 B), H_2O_2 did not significantly influence transfer of fatty acid from neutral lipid to PE.

Effects of removal of radioactivity from the superficial pool of fatty acids upon incorporation of fatty acid into PE. Cells were prepared as described in Methods section of 5 C and incubated (Fig. 3). After replacement of the surface pool of fatty acid with nonradioactive fatty acid, no increment of palmitic acid-^{3}H could be detected in PE during incubation with or without H_2O_2. This result may be contrasted with the ex-

TABLE I

Prelytic Phospholipid Alterations in E-Deficient Cells Incubated with H_2O_2

Patients	Total lipid phosphorus	Individual phospholipids*			
		PE	PS-PI	PC	SP
1	84	63	97	99	100
2	100	100	98	99	100
3	85	70	99	98	100
4	90	80	100	89	100
5	100	100	100	99	100
6	100	100	100	100	100
7	94	88	97	100	100
8	100	100	100	99	100

The washed red cells were incubated in KHB with H_2O_2 (1% final concentration) for 1 hr. The media were removed, and the cells were washed with saline before lipid extraction and analysis. All results are expressed as the percentage of the pre-incubation values of total phospholipid/milliliter red cells. See Methods section 5 A.

* PE, phosphatidylethanolamine; PS-PI, phosphatidylserine, phosphatidylinositol; PC, phosphatidylcholine; SP, sphingo-myelin.

periment presented in Fig. 2 A in which the superficial pool was retained.

Specific activity of saturated and unsaturated PE fatty acid. The change in specific activity of typical PE fatty acids which occurred when red cells were in-

TABLE II

Specific Activity of Phosphatidylethanolamine after Incubation with Hydrogen Peroxide for 30 min

	Saturated fatty acid		Unsaturated fatty acid	
	H_2O_2	Buffer	H_2O_2	Buffer
	cpm/μg lipid P		*cpm/μg lipid P*	
E deficient	3,400	400	340	185
Normal	365	300	170	170
E deficient	17,500	12,900	470	390
Normal	1,000	1,400	140	135
E deficient	4,730	3,180	580	550
Normal	1,300	1,300	170	145
E deficient	5,600	900	640	580
Normal	1,110	1,060	250	220
E deficient	7,400	1,500	970	670
Normal	1,600	1,500	300	290

Red cells were preincubated with saturated fatty acid (palmitic-9, 10-³H) and unsaturated fatty acid (arachidonic-1-¹⁴C), washed with saline, and reincubated with hydrogen peroxide. The results are expressed as counts per minute/microgram lipid phosphorous and represent the average of duplicate determinations on blood from five separate patients and controls. See Methods section 5 A.
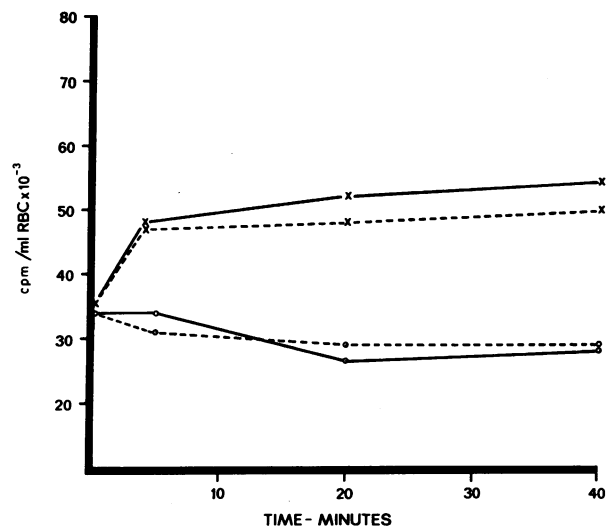

FIGURE 3 The influence of the albumin extractable lipid pool on changes in phosphatidylethanolamine and neutral lipid fatty acid during incubation in hydrogen peroxide. Vitamin E-deficient cells were preincubated with palmitic-9,10-³H for 2 hr in the presence of glucose. They were washed with saline and defatted albumin to remove the superficial free fatty acid pool (14). A nonradioactive superficial pool was then reintroduced by incubation in autologous serum, and the cells were washed in saline before final reincubation in KHB (----) or in KHB and 2% hydrogen peroxide (———). The red cell lipids were extracted, isolated by TLC, and counted. The radioactivity isolated is plotted as counts per minute/milliliter RBC. ○ = counts in neutral lipid; × = counts in PE. See Methods section 5 C.

cubated with H_2O_2 for 30 min is seen in Table II. The changes in specific activity of both saturated fatty acid (palmitic acid-³H) and an unsaturated FA (arachidonic acid-¹⁴C) in five separate experiments are recorded. In the E-deficient cell, incubation with H_2O_2 caused a much greater increase in specific activity of the satu-rated fatty acid than of the unsaturated fatty acid. In normal cells, the specific activity of both unsaturated and saturated fatty acid was not significantly changed during the incubation in H_2O_2.

The effect of H_2O_2 on the distribution of fatty acid in PE. The influence of H_2O_2 on the predominant fatty acid composition in PE in a typical experiment is seen in Table III (see Methods sections 3 and 5 B). Thymol was added at 30 min in an effort to prevent further changes due to lipid peroxidation. After exposure to H_2O_2, the concentration of palmitic acid was increased in the E-deficient cells while the concentration of arachi-donic acid was decreased. These alterations were not present in the phosphatidylethanolamine fatty acids of normal cells incubated with H_2O_2. Similar results were obtained in four other incubations which are not shown. While the initial concentrations of fatty acids were

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TABLE III

**[Percentage Distribution of Predominant Fatty Acids in
E-Deficient and Normal Cell Phosphatidylethanolamine
with and without Incubation with H_2O_2**

	Patient*		Control	
	Buffer	H_2O_2	Buffer	H_2O_2
16:0	17	24	14	15
18:0 + 18:1 DMA	16	18	13	12
18:1	20	21	18	17
18:2	7	6	6	6
20:4	20	13	23	24
22:4-6	15	11	18	16

After incubation of red cells in buffer with 1% H_2O_2 for
45 min, saturated thymol was added to inhibit further
peroxidation. Hemolysis had not occurred at this time. After
extraction and separation, PE fatty acids were determined by
TLC. Total PE did not change during the time of these
observations ($\pm 4\%$). No major or consistent changes in
saturation were detected in the remaining minor fatty acids.
The experiment shown here is representative of five similar
incubations. See Methods sections 3 and 5 A.

* All data are expressed as percentage of total fatty acids
present (determined from the sum of the gas chromatography
peaks corrected for molecular weights).

variable due to the differing ages and types of disease
of the donor patients, in all cases there was an increase
in the amount of 16:0 and 18:0 saturated fatty acids
and a decrease in polyunsaturated fatty acids after ex-
posure to H_2O_2.

DISCUSSION

In 1952, Rose and Gyorgy (13) described the hemo-
lytic effect of H_2O_2 on vitamin E–deficient red blood
cells. The mechanism of this event is still not completely
understood. In the absence of vitamin E or a similar
antioxidant, phosphatide unsaturated fatty acids are
peroxidized with resultant schism of the fatty chains
(20). The residual esterified short acyl fragment would
then tend to escape from its previously protected hydro-
phobic environment (21). It would then be especially
susceptible to removal by an endogenous erythrocyte
phospholipase (22). The resultant lysophosphatidyletha-
nolamine might then be lost to the medium due to its
high water solubility or it might be reacylated within
the membrane (14, 23, 24). PE containing a high per-
centage of unsaturated fatty acids is particularly sus-
ceptible to peroxide alterations (6). In vitamin E–de-
ficient erythrocytes from rats and patients with A-β-
lipoproteinemia, prelytic loss of PE has been associated
with hemolysis (3, 4). In addition, products of the
peroxidation of unsaturated fatty acids also inhibit sul-

fydryl-containing enzymes and may potentiate mem-
brane damage (25).

The present study was undertaken to investigate
further the influence of H_2O_2 on human vitamin E–
deficient red cells. In addition to examining quantitative
changes in individual phosphatides, the influence of
H_2O_2 on the incorporation and turnover of fatty acids
within red cell lipid classes, specifically PE, was de-
termined.

Studies on red cells from six vitamin E–deficient
patients indicated that prelytic loss of phospholipid was
not essential for hemolysis during incubation with H_2O_2
(Table I). When phospholipid loss was present, it was
confined to PE. The degree of vitamin E deficiency in
previously reported studies in rodents may have been
more profound than that seen in the children in this
study and may account for the observed consistent pre-
lytic loss of PE in those studies (3).

The incorporation of fatty acids into vitamin E–de-
ficient red blood cells without H_2O_2 was greater than that
into normal cells. This difference may be explained by
red cell age alone. The reticulocyte counts in the E-
deficient cells ranged from 4% to 6% while the reticu-
locyte counts in the normal cells were 2% or less.
Young red cells incorporate fatty acid at a greater rate
than do old cells (26). However, H_2O_2 *further* stimu-
lated the incorporation of fatty acid into E-deficient
cells but had no effect on the incorporation of fatty
acid into normal cells (Fig. 1) (27) or into normal
reticulocytes (data not shown). Fig. 1 B shows that
the H_2O_2-stimulated increase in fatty acid incorporation
was confined to PE. Since the quantity of PE was not
changed, the increase in radioactivity indicates an in-
creased turnover of fatty acid in PE.

The data in Fig. 2 and Table II demonstrate that
there is a transfer of both saturated and unsaturated
fatty acid from neutral lipid into PE. The data in Fig.
3 show that this transfer is dependent upon the neutral
lipid precursor pool (14). This transfer is stimulated
in the E-deficient cell by incubation in H_2O_2 and can
occur before hemolysis. The final accumulation of satu-
rated fatty acid is far greater than unsaturated fatty
acid (Table II). This difference may be explained by
the specific susceptibility of unsaturated fatty acid to
H_2O_2 in contrast to saturated fatty acid: while reacyla-
tion of both unsaturated and saturated fatty acid may
occur, the newly acquired unsaturated fatty acid con-
tinues to be at risk to ongoing peroxidation, whereas
the newly acquired saturated fatty acid is resistant.
Hence, saturated fatty acid accumulates. The cumula-
tive effect of these processes is a remodeling of red cell
PE in terms of its fatty acid composition.

Fatty acid analytic data (Table III) confirmed these
radioactivity measurements by showing a decrease in

the percentage of the predominant unsaturated fatty acids in PE after incubation with H_2O_2. Since the amount of total phospholipid did not change during these experiments, these percentage shifts reflect changes in the absolute amounts of PE fatty acid as well. The extent of these changes is surprisingly high for the brief period of incubation used. This may be due to both the general stimulation of acylation reactions previously observed in buffer systems (23, 24) and to the specific stimulation of PE fatty acid turnover observed under the influence of H_2O_2 here (see Fig. 1 B). Also, remotely, the thymol treatment used to inhibit peroxidation may have not totally terminated the process, and some turnover may have continued until the time of lipid extraction. All of these factors limit quantitative extrapolation from the extent of these in vitro observations to in vivo situations. However, the qualitative trend indicated by these observations is entirely consistent with the previous in vivo observations of Jacob and Lux (3).

When E-deficient cells previously labeled with radioactive fatty acid were washed with defatted albumin and reincubated in serum to reestablish a nonradioactive superficial pool, the transfer of radioactively labeled fatty acid from neutral lipid into PE was not observed. As expected, this was probably due to the removal of radioactivity from the superficial fatty acid pool and indicates that this pool provides the fatty acid for the repair process stimulated by H_2O_2.

Alterations in the permeability of experimental lipid membranes can occur with qualitative or quantitative changes in their lipid components. Studies performed on artificially prepared lipid micelles have shown increased sodium permeability when the content of linoleic acid was decreased (28). Variations in cation permeability in mammalian red cells may also reflect specific changes in fatty acid concentration (29). By analogy, the alterations seen here in the composition of fatty acid in erythrocyte PE might be expected to alter red cell permeability. This expectation has been born out by observations of increased permeability and decreased cation content in vitamin E–deficient erythrocytes after exposure to hydrogen peroxide but before hemolysis. These changes, which occur simultaneously with the membrane lipid changes just described, will be the subject of a further report.

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