Membrane Lipid Peroxidation and Its Prevention[1]

JAMES F. MEAD, Laboratory of Nuclear Medicine and Radiation Biology, University of California, Los Angeles, 900 Veteran Ave., Los Angeles, CA 90024

ABSTRACT

The study of membrane peroxidation has been carried out using model systems consisting of a polyunsaturated fatty acid monolayer and an unsaturated phosphatidylcholine bilayer system. It has been shown that the major products of lipid peroxidation in monolayer and mixed saturated-unsaturated liposomes are epoxides, hydroxy-epoxides and their hydrolysis products. These same products are found in lungs of rats, particularly after 24 hr of breathing 6 ppm NO_2. In all cases, inclusion of tocopherol introduces a lag period during which little peroxidation occurs.

Today, I would like to say something about our present results and future plans and give one of the only examples in my experience in which information gained with the simple model systems has proved to be consistently accurate in predicting events in a series of more complex systems. Usually, this is merely the way you hope it will be.

When we were tracing the metabolic pathways of the essential fatty acids, we were always looking over our shoulders with the uneasy feeling that something was accompanying us step by step. It was only after Max Horwitt made us turn around and face it squarely that we recognized an old acquaintance—autoxidation. Was it possible that, by building up the tissue polyunsaturated fatty acids with all the obvious and supposed benefits that would accrue, we were creating susceptibility to reactions that would increase tissue destruction, promote certain diseases and hasten senility?

Impressed by this possibility, many scientists, from physical chemists to physicians, have attempted to answer this question, but a direct answer still can not be given. It is quite difficult to measure peroxidation in living animals and any tissue disruption brings about a peroxidative reaction which masks anything that might have occurred in the intact animal. Another difficulty is that we have assumed that the same radical propagated chain reaction that has been thoroughly studied in neat unsaturated oils would also take place in tissues. Figure 1, a simplified version of this complex reaction, illustrates the reaction's complexity and calls attention to its major product, fatty acyl hydroperoxide.

We were impressed by the probability that in the ordered arrangement of unsaturated fatty acid chains present in the membrane bilayer (Fig. 2), a radical propagated peroxidation could readily take place.

Proof of this assumption has been difficult because of the complexity of cell membranes. Most investigators have used model systems that isolate and simplify certain aspects to permit the investigator to ask intelligent questions and to understand the answers.

In our laboratory, we have used a series of model systems of increasing complexity. The simplest is a monolayer of polyunsaturated fatty acid, usually linoleic acid, adsorbed on silica. Our hope was that this model would exemplify the ordered arrangement of unsaturated fatty acids in the membrane bilayer and thus would show similar properties in peroxidation reactions. A schematic representation of this monolayer is shown in Figure 3. The ordered arrangement of linoleate chains, more or less parallel, with unsaturated centers more or less in a plane, is an ideal arrangement for a chain reaction. Also included is a saturated fatty acid interposed between the unsaturated chains and schematic cholesterol and α-tocopherol. I will discuss these constituents later.

We know that the fatty acids in the monolayer system are bound noncovalently to isolated silanol groups (Fig. 4)

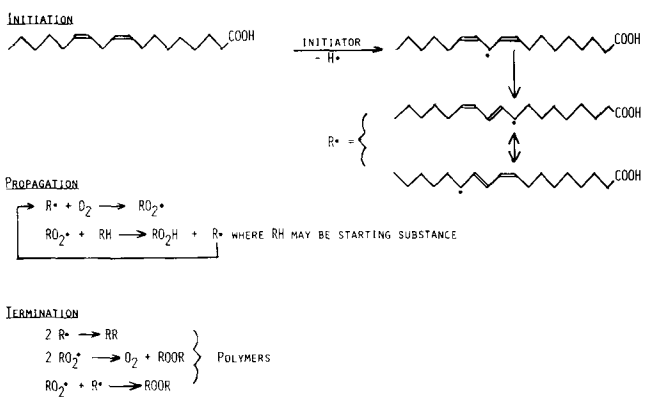


FIG. 1. The mechanism of linoleic acid autoxidation in bulk phase.

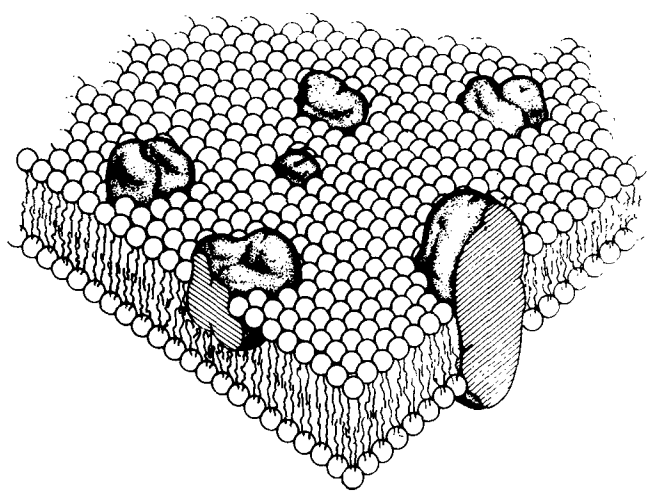


FIG. 2. Schematic view of the lipid bilayer membrane. (From S.J. Singer and G.L. Nicholson, Science 175:720 [1972] copyright [C] 1972 by the American Association for the Advancement of Science).

[1] Presented as the acceptance address for the Award in Lipid Chemistry, April 28, 1980, during the ISF/AOCS World Congress.

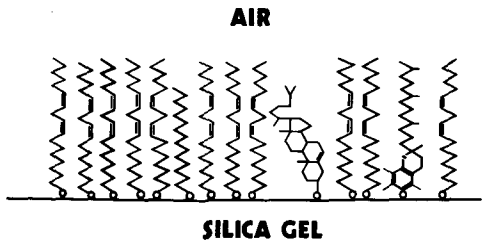


FIG. 3. Schematic view of a linoleic acid monolayer on silicic acid also showing one molecule each of palmitic acid, cholesterol and α-tocopherol.

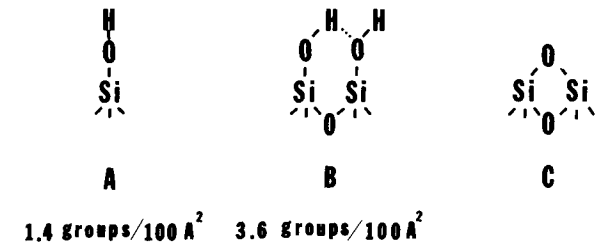


FIG. 4. Types of silicon-oxygen bonds at surface of silica gel.

making the distance between them close to what it is in the phospholipid, ca. 5 Å (1). We have also found that, with such an arrangement, the classical autoxidation scheme may not hold. Information gained with this simple system predicts findings in all our other models and in the living animal.

When peroxidation is initiated and the reaction followed by disappearance of linoleate, first order kinetics are observed (Fig. 5) (2). Since the classical autoxidation reaction has kinetics that are much more complex, it was apparent to us that the two are different. This was confirmed when, in both the monolayer and the neat systems, the reaction was allowed to proceed to the same extent and the products were examined. Figure 6 shows that although hydroperoxide is the major product of the bulk phase reaction, it is barely present in the products from the monolayer reaction. Instead, twin spots in a less polar position appeared. We recognized these spots from previous work as belonging to fatty acid epoxide and, using thin layer chromatography (TLC), gas liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS), confirmed them as the isomeric epoxy octadecenoic acids. We rationalize the production of these compounds as a direct result of the ordered arrangement of the unsaturated chains in which the peroxy radical on one chain transfers an oxygen to the double bond of a neighboring chain (Fig. 7). Any olefinic substance present in this monolayer is epoxidized during the reaction, including oleic acid, which does not form epoxides alone, and cholesterol, in which the α-5,6-epoxide is a known carcinogen. It is notable that the aromatic hydrocarbon epoxides, formed enzymatically in the tissues, are the true carcinogenic derivatives of the known carcinogenic hydrocarbons.

Real membranes rarely are formed solely of linoleate-containing phospholipids and the effect of saturated fatty acids on the monolayer was notable. The prediction was that, with the resistant saturated chains interposed between the reactive unsaturated chains, the rate of peroxidation would be decreased, provided that the chains were long enough to interfere with oxygen transfer. Figure 8 shows that the longer chain fatty acids are much more effective in reducing the formation of epoxide than are the short chain homologs (3).

When the rate of the reaction is decreased either by addition of saturated fatty acids or by interposing space between the unsaturated chains by decreasing the ratio of linoleate to silica, fatty acid epoxide decreases and a more polar product becomes prominent. This product was found, using the same analytical techniques, to be composed of the isomeric 11-hydroxy, 9 or 12 epoxy octadecenoic acids (2). We attribute the production of these compounds to the difficulty of intermolecular oxygen transfer under these conditions with the result that it is transferred intramolecularly (Fig. 9). The hydroxyepoxides are the most potent carcinogens derived from the aromatic hydro-

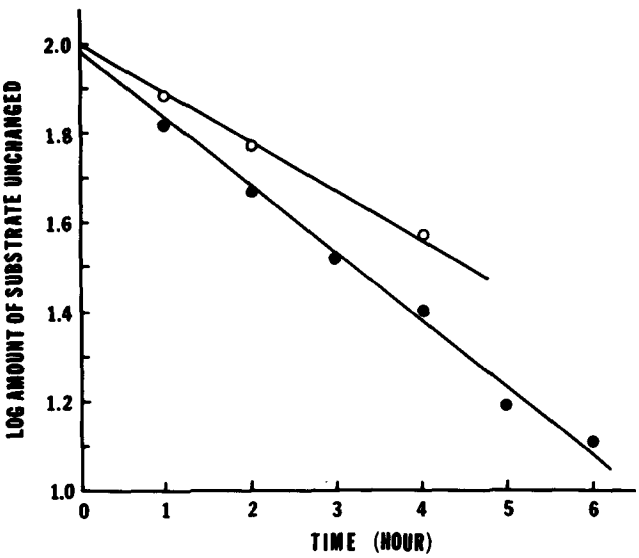


FIG. 5. Semilog plot of decrease of linoleic acid (closed circles) or linolelaidic acid (open circles) during autoxidation of the monolayers with time.

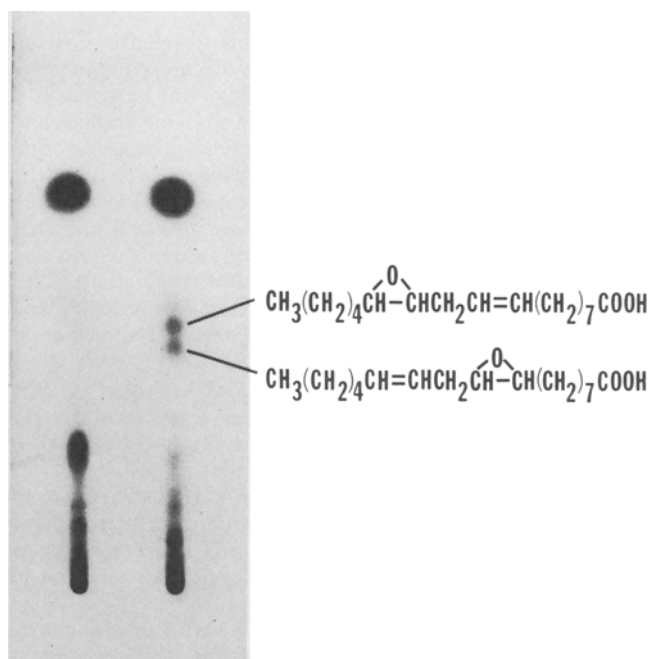


FIG. 6. Thin layer chromatogram of products of autoxidation of linoleic acid in bulk phase (left channel) or monolayer (right channel). Extent of oxidation 60% in both cases.

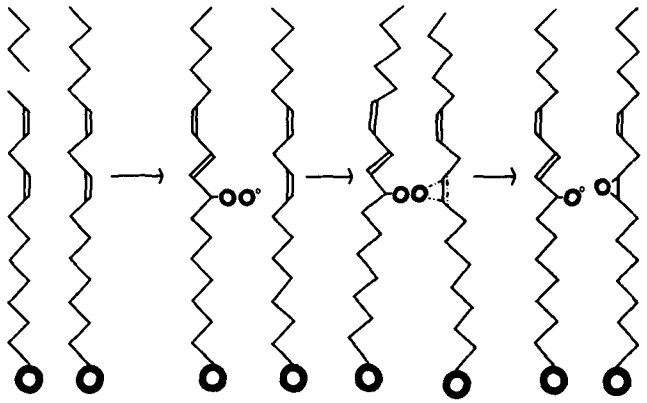


FIG. 7. Schematic diagram of proposed mechanism of oxygen transfer from hydroperoxy radical to adjacent unsaturated fatty acid in the monolayer.

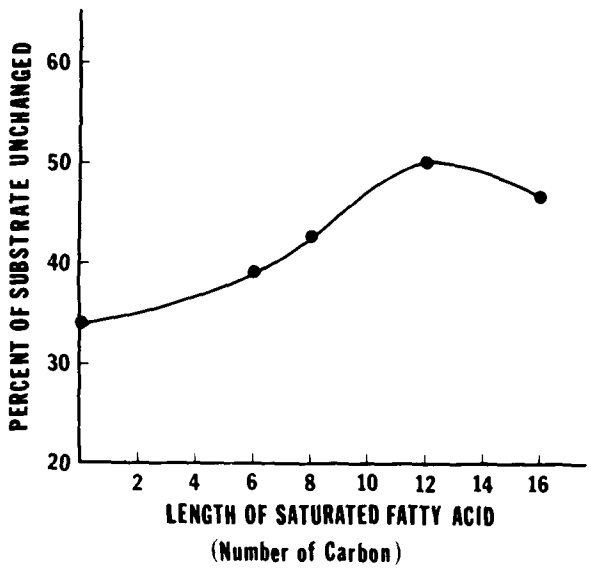


FIG. 8. Inhibition of autoxidation of linoleic acid in monolayer by saturated fatty acids of different chain lengths.

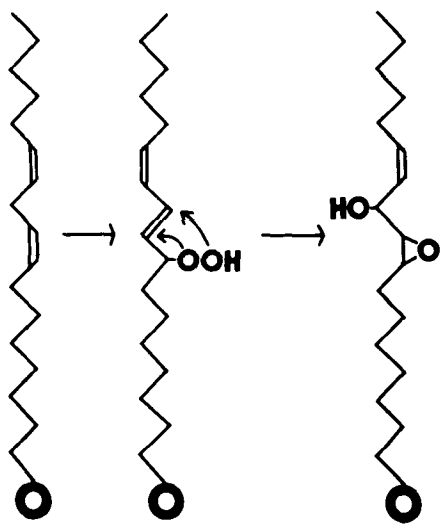


FIG. 9. Schematic diagram of proposed mechanism of intramolecular transfer of oxygen and hydroxyl in 9-hydroperoxy 10,12-octadecadienoic acid.

carbons.

Under these circumstances, prevention of these reactions assumes importance and the use of antioxidants is suggested. When the chief membrane antioxidant, α-tocopherol (Vitamin E) was included in the monolayer, an induction period resulted during which little oxidation of linoleate occurred (4). When the tocopherol had decreased to 10% of its starting proportion (to 0.004 mol %), oxidation occurred at the same rate as if the antioxidant had not been there at all. γ-Tocopherol produced an induction period longer than that of α-tocopherol but in some cases seemed to permit somewhat more oxidation during the induction period. A synthetic antioxidant synthesized in our laboratory, 3-(ω-carboxynonyl)-4-methoxy-5-pentylphenol, represented a more extreme case of the same type of antioxidant action in that it produced a long induction period during which slow oxidation took place (Fig. 10). A combination of FAHQ and α-tocopherol, theoretically the perfect antioxidant, gave a very long, oxidation-free lag period. It is interesting that tocopherol continues to protect the fatty acids from oxidation even at 10% of the initial concentration of 0.04 mol %. This means that one tocopherol molecule is protecting about 20,000 molecules of fatty acid.

A model system closer to the cell membrane is the bilayer liposome formed by sonicating aqueous suspensions of lecithin. When highly unsaturated soybean lecithin was used and peroxidation induced, the major product was the hydroperoxide, as predicted from the classical bulk-phase autoxidation, actually isolated as the corresponding hydroxy compounds. However, when increasing proportions of the saturated dipalmitoyl lecithin were added, the kinetics and products changed to resemble those of the monolayer (5) (Fig. 11). Figure 12 shows that, in the presence of the saturated lecithin, the proportions of hydroxy-epoxy fatty acids and their hydrolysis products, the trihydroxy acids, have increased greatly. From the results with the monolayer, it would appear that the saturated fatty acids prevent intermolecular oxygen transfer so that, in this model, as well, intramolecular transfer takes place.

The next more complex membrane system, the liver microsomal membrane, has been used so far as a test for the effect of NO_2 as an initiator of peroxidation and the effectiveness of tocopherol in preventing the reaction. Microsomal preparations from vitamin-E-deficient or vitamin-E-supplemented rats were incubated in the presence of iron-ascorbate, a "normal" initiator of tissue peroxida-

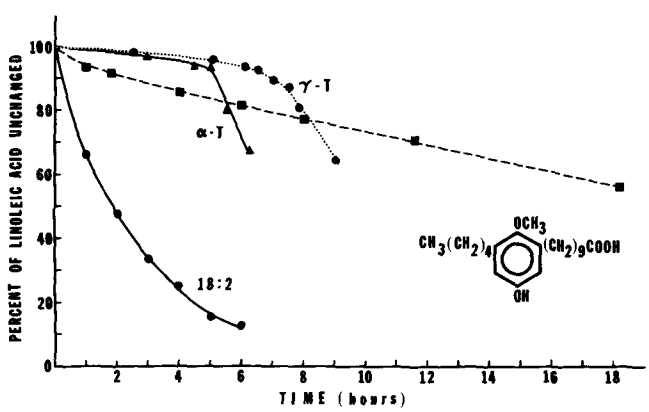


FIG. 10. Effect of antioxidants on autoxidation of linoleic acid monolayers. Closed circles, solid line—no antioxidant; closed circles, dotted line—0.05 mol % γ-tocopherol; closed squares, dashed line—0.45 mol % FAHQ.

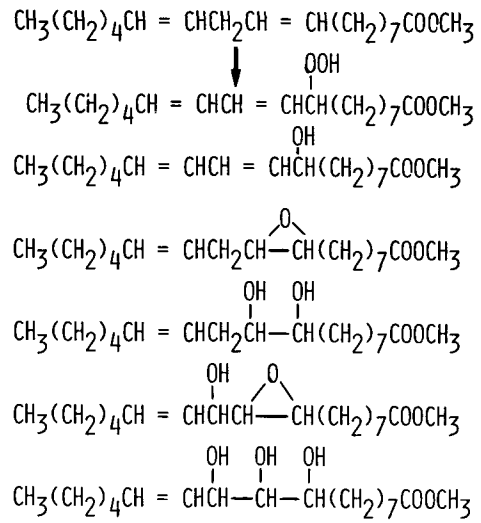


FIG. 11. Autoxidation products from soybean PC.

tion (6), or under 12 ppm of NO_2. Peroxidation was assessed by measuring the loss of polyunsaturated fatty acid (docosahexaenoic or arachidonic) and the concentration of tocopherol was also measured (7). In the microsomes from the tocopherol-supplemented rats, the fatty acids were well protected from oxidation by the iron-ascorbate system, but in the deficient microsomes, considerable oxidation occurred. NO_2 apparently did not affect the fatty acids, but resulted in a faster rate of decrease of tocopherol content, particularly in microsomes from deficient rats. This relationship can be seen even more clearly in the monolayer system, which already contains an initiating agent, possibly iron. As was seen before, tocopherol protects the fatty acid from peroxidation, even at concentrations as low as 0.005 mol %. However, in the presence of NO_2, this protection is lost and the fatty acid is readily oxidized. The reason for this loss is clear after a check of the effect of NO_2 on tocopherol.

The tocopherol decreases so rapidly that it affords almost no protection to the fatty acid, which is then oxidized by the usual initiation in the silica (7) (Fig. 13). Our conclusions are that NO_2 is an effective initiator of oxidation of tocopherol and that, once the antioxidant has been reduced below a minimal level, peroxidation of membranes occurs.

At this point, we may ask whether any of the information gained in the various model systems has any bearing on the case of the whole animal or of human subjects.

In experiments with rats breathing either air or air with 6 ppm NO_2, extraction and analysis of the lung tissue lipids and the lung lavage lipids revealed the same fatty acid and cholesterol epoxides that had been seen in the model systems. The most interesting and revealing aspect of these findings is seen in the location of the increased concentrations of epoxides produced by NO_2. Figure 14 shows a significant increase in the lung tissue phospholipid epoxide and in the cholesterol, but none in the triglyceride. This would have been predicted from the model system since the membrane lipids should be the most readily affected. However, Figure 15 shows that, in the lung lavage, it is not the phospholipid epoxide that increases but that in the triglyceride. There is a possible explanation for this peculiar finding. Among the many defense mechanisms against peroxidative reactions available to the cell might be an increased rate of removal of oxidized fatty acids from the membrane phospholipids and its disposal in some safe manner. The chief enzyme obviously involved in the hydrolysis of an oxidized fatty acid from a phospholipid would be phospholipase A_2, since the unsaturated fatty acid and, consequently, the oxidized fatty acids should be in the 2-position. This was checked by comparing the rate of hydrolysis of [1-^{14}C] oleic acid and its product, 9,10-epoxystearic acid from the 2-position of phosphatidylcholine. Both soluble phospholipase A_2 and the particulate enzyme from lung microsomes removed the oxidized fatty acid from the phospholipid at twice the rate of its unsaturated precursor. The appearance of the product in the triglyceride fraction is not explained by these experiments. Moreover, lung homogenates that normally incorporate fatty acids into triglycerides do not do so with 9,10-epoxystearate. Clearly, another explanation is necessary and at present the only reasonable one is that phospholipase C may also be more active with an oxidized phospholipid and that the resulting diglyceride may be released from the membrane and acylated to form the triglyceride. Such a reaction may be unnecessary, however, in the light of another finding on the tissue's defense mechanisms. The principal enzyme responsible for disposal of epoxides in the tissues is epoxide hydrolase, which converts the epoxide to the presumably harmless dihydroxy product. Rate studies of the reaction of epoxide hydrolase with epoxidized fatty acid or with the same fatty acid incorporated into a phospholipid have revealed that the free fatty acid reacts about 10 times more rapidly than does the phospholipid. Thus, only after release is the fatty acid epoxide hydrolyzed at an

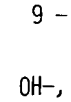
	SOYBEAN PC	SOYBEAN PC + DPL
	3.9	4.8
13 - OH-	45.3	10.8
9 - OH-	33.3	16.6
	1.0	12.3
DI - OH-	3.0	3.8
COMP. X	4.9	6.5
TRI-OH-	6.1	45.2

FIG. 12. Yields of various autoxidation products in two systems (%).

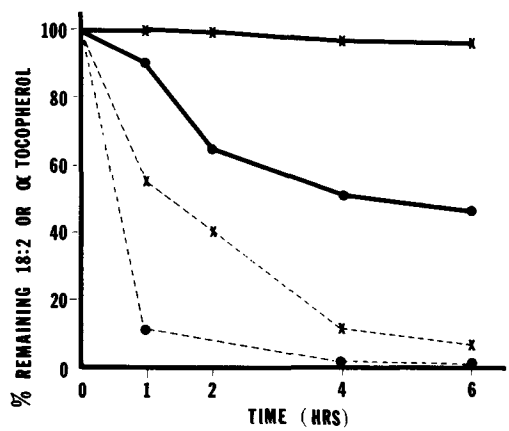


FIG. 13. Oxidation of linoleic acid (—) and α-tocopherol (---) from the monolayer of DTPA-treated Silica Gel G under air (x) or 10 ppm NO_2 (•) at 60 C.

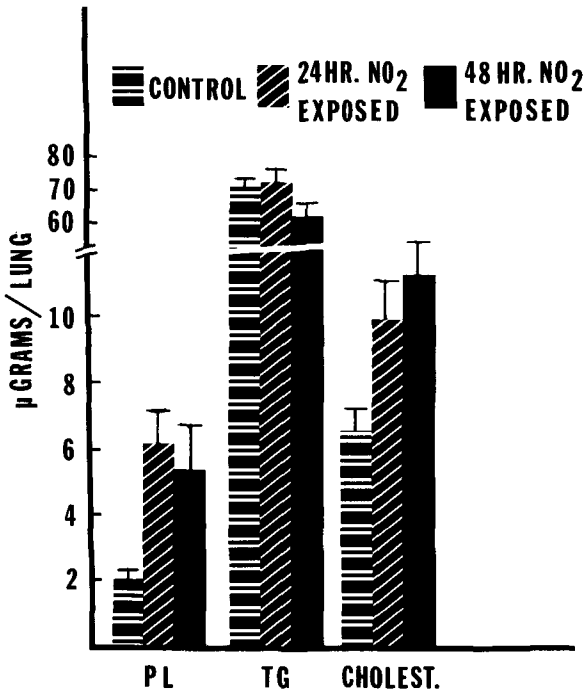


FIG. 14. Concentrations of fatty acid and cholesterol epoxides in different fractions of lung tissue lipids following 24 or 48 hr exposure of rats to filtered air or 6 ppm NO_2.

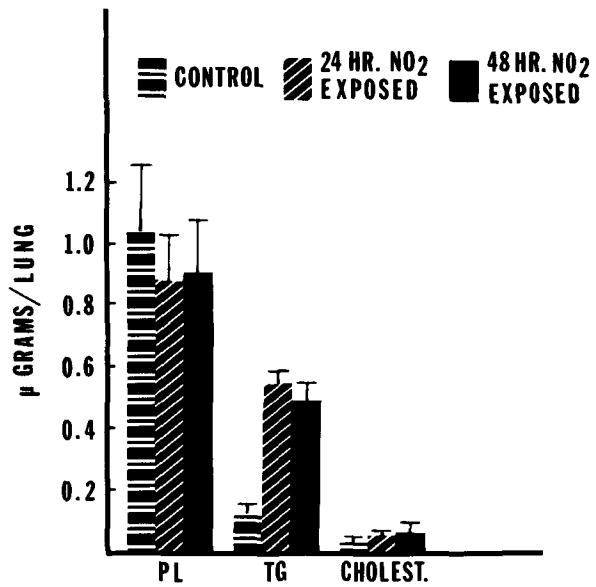


FIG. 15. Concentrations of fatty acid and cholesterol epoxides in different fractions of lung lavage lipids following 24 or 48 hr exposure of rats to filtered air or 6 ppm NO_2.

appreciable rate to be disposed of as the dihydroxy fatty acid.

A consideration of the many protective devices available to the tissues against this type of oxidation leaves us with the satisfaction that we are well protected but with the apprehension that the reaction is a real danger. These studies have shown that the defense most readily manipulated by the individual is the availability of tocopherols (vitamin E). As a matter of fact, a comparison of lung tissue tocopherol with epoxide content in NO_2-exposed rats reveals that they are inversely related. Perhaps, in these perilous times when everything we eat, drink or breathe seems to cause cancer or promote aging, we should use any device at our disposal to preserve us for a heroic death in an automobile accident.

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