Vitamin E: The Biological and Environmental Antioxidant

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Trace amounts (ppm) of the air pollutants O_3 and NO_2 rapidly oxidize polyunsaturated fatty acids. Phenolic antioxidants retard this oxidation. Vitamin E decreases the acute toxicity of both O_3 and NO_2. On continuous exposure to 1.5 ppm of O_3 the LT_{50} for vitamin E-depleted rats was 8.2 days, compared to 18.5 days for continuously supplemented rats. Similarly the LT_{50} for depleted rats exposed to 33 ppm of NO_2 was 11.1 days *vs.* 17 days. Exposure to 0.5 ppm of O_3 also accelerated the depletion of vitamin E from erythrocytes of exposed animals in 23 days *vs.* 36 days for unexposed animals. The polyunsaturated fatty acid content of lung tissue significantly declined in rats fed a constant fatty acid composition diet free of vitamin E and/or exposed to NO_2. O_3 exposure decreased the oleic and linoleic acid content but increased the arachidonic acid content. These changes may be complex responses of the lung to increased oxidant stress, as shown by depression of serum reduced glutathione and tissue sulfhydryl compound content, or may be related to other metabolic roles of vitamin E in the biosynthesis of polyunsaturated fatty acids.

We have chosen as our working hypothesis the theory that vitamin E (α-tocopherol) functions *in vivo* primarily as a lipid antioxidant. Such a general role fits best with the diversity of vitamin E deficiency syndromes: fetal resorption (Evans and Bishop, 1922); muscular dystrophy (Matill and Golumbic, 1942); liver necrosis (Schwarz, 1944); encephalomalacia (Dam and Glavind, 1938); and exudative diathesis (Schwarz *et al.*, 1957). Other antioxidants substitute for tocopherol eliminating the symptoms of tocopherol deficiency, particularly fetal resorption (Draper *et al.*, 1958) and muscular dystrophy (Draper and Csallany, 1959; Draper, 1959).

Witting and Horwitt (1964) and Witting (1967) studied the oxidation of polyunsaturated fatty acids (PUFA) predicting the effect of the fatty acid composition of the diet upon the rate of appearance of creatinuria as a symptom of tocopherol deficiency. Witting (1967) calculated that 125 μg of PUFA esterified to muscular phospholipids were oxidized before creatinuria occurred. According to this interpretation, selective oxidation of higher PUFA should occur and, in fact, Witting reports a preferential decline during tocopherol depletion.

Tocopherol status has been related to the toxicity of oxidizing environments. Oxygen toxicity, in particular, appears especially sensitive to tocopherol, sulfur amino acid, and selenium dietary intakes (Shaw *et al.*, 1972). Alterations in erythrocyte fatty acid compositions resulting in more saturated and less unsaturated fatty acids have been interpreted as due to selective oxidation of higher PUFA.

The air pollutants, O_3 and NO_2, are strong oxidizing agents commonly found in urban air. While the pathology of O_3 and NO_2 has been well described, a mechanistic interpretation of their toxicity has been lacking. A common mechanism of action through lipid oxidation has been proposed which would account for their radiomimetic properties (Menzel, 1970; Roehm *et al.*, 1971a). NO_2 alteration of lung lipids does, in fact, occur (Roehm *et al.*, 1971b) and the toxicity of high O_3 concentrations is greater for tocopherol-depleted rats (Roehm *et al.*, 1971a; Goldstein *et al.*, 1970). We shall report here our studies of these phenomena which suggest that the individual's response to O_3 and NO_2 is a function of this dietary intake of tocopherol and unsaturated fatty acids. Oxidizing air pollutants are at once probes of the function of tocopherol as an antioxidant *in vivo* and, perhaps, the molecular pathology of obstructive lung diseases.

MATERIALS AND METHODS

Animals and Diets. Male weanling rats of the Sprague-Dawley strain were fed a semi-purified basal diet (Table I) containing 7% randomized triglycerides. These diet triglycerides were prepared from molecular distilled fatty acids by esterification in 4% sulfuric acid–methanol, followed by rearrangement with triacetin (Whitting and Horwitt, 1964). The resultant triglyceride, containing over 50% linolenic acid, was stored under nitrogen at −20°C, as was the diet which was compounded twice weekly. The synthetic triglyceride was chosen to provide the greatest tissue unsaturated fatty acid composition and hence greater tissue susceptibility to oxidation (Witting and Horwitt, 1964).

Animal Exposures. For each exposure study, two groups of ten rats each were housed in a single Plexiglas gas mixing glove chamber of 340-l. capacity and air flow of 28.3 l./min. Ozone was supplied by a silent arc generator (Bonner, 1953) and was determined periodically (Saltzman and Gilbert, 1959). Exposure was continuous.

At various time intervals animals were withdrawn from the chamber, anesthetized with pentobarbital (50 mg/kg) intraperitoneally, and the heart–lung block was excised. The lung was then trimmed of the heart, thymus, and esophagus and weighed. Endobronchial lavage was performed on each lung by four successive washes with deoxygenated saline to an approximate total wash of 10% of the body weight. The lung tissue and saline lavages were stored at −20°C prior to analysis.

Lipid Analysis. Lung tissue was extracted in 20 vol of of chloroform:methanol (2:1) in a Duall tissue homogenizer and the lipid was isolated according to the method of Folch *et al.* (1957). The lavage lipids were isolated by first evaporating the saline under reduced pressure at 50°C to an approximate volume of 1 ml and proceeding as with the tissue.

The fatty acid composition of the extracted lipids was determined by first reacting these lipids with 4% H_2SO_4 in anhydrous methanol for 1 hr at 100°C. The resultant methyl esters were determined using a Packard Model 7401 gas

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Table I. Experimental Diet

Ingredient	Weight %
Casein	22.8
Salt mixture 445 (NRRL)	3.5
Dextrose	65.9
Lipids[a]	7.0
Choline dihydrogen citrate	0.3
Vitamin mix[b]	0.3
Vitamin D	2400 IU/kg
Vitamin E (*dl*-α-tocopherol acetate)	100 mg/kg

[a] Synthetic triglyceride. Fatty acid composition (weight %): 16:0 = 6.45; 18:0 = 4.45; 18:1 = 21.30; 18:2 = 17.22; 18:3 = 50.58. [b] Vitamin mix contains (mg per 0.3 g) inositol, 11.1; *p*-aminobenzoic acid, 11.1; D-calcium pantothenate, 6.2; 2-methyl-1-4-naphthoquinone, 5.0; niacin, 10.0; thiamin · HCl, 2.2; pyridoxine · HCl, 2.2; riboflavin, 2.2; folic acid, 0.2; biotin, 45 μg; vitamin B_{12}, 3 μg; vitamin A, 2400 I.U.; and dextrose to 0.3 g.

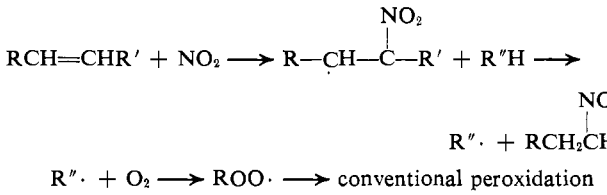
chromatograph equipped with a dual flame ionization detector. The column used was 6-ft × 2-mm i.d. glass packed with 15% HiEFF-IBP on 100/120 mesh Gas Chrom P, and was operated at 180°C and 16 psig inlet pressure. Quantitative data were calculated by $R_t \times h$ corrected to weight percent (Ackman and Sipos, 1964). Analyses were checked using methyl ester standards (Hormel Institute, Austin, Minn.).

Erythrocyte Hemolysis. Plasma tocopherol was estimated by the dialuric acid hemolysis of erythrocytes. This method is an index of the peroxidizability of cell membranes. Blood was withdrawn by incision of the tail vein and subjected immediately to hemolysis (Friedman *et al.*, 1958). Hemolysis was estimated colorimetrically at 415 nm.

Sulfhydryl Determinations. Blood-reduced glutathione was determined immediately on samples obtained by cardiac puncture (Buetler *et al.*, 1963). Tissue sulfhydryl compound content was determined by the method of Sedlak and Lindsay (1968).

RESULT

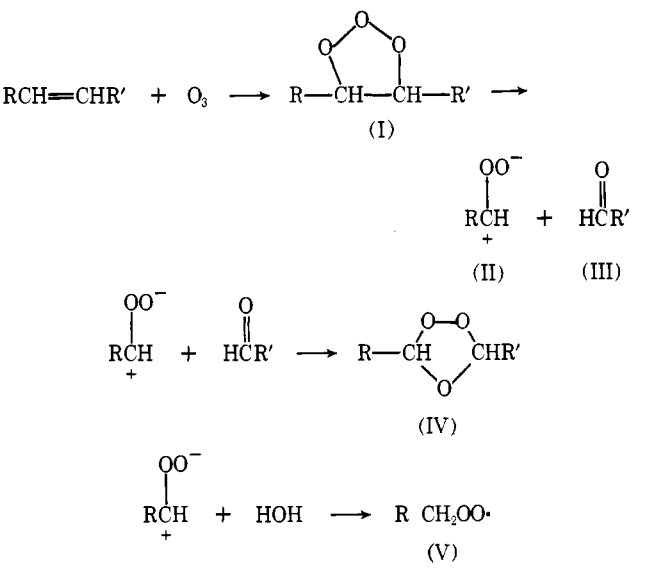
***In Vitro* Oxidation of Polyunsaturated Fatty Acids.** Trace amounts of O_3 and NO_2 initiate the rapid oxidation of PUFA; thin films of methyl linoleate oxidize to produce malonaldehyde as shown in Figure 1. The rate of oxidation is proportional to the NO_2 concentration, and an induction phase, as occurs with the spontaneous air oxidation, does not occur. The rate of NO_2 oxidation is retarded initially by the phenolic antioxidants, butylated hydroxyanisoles (BHA), butylated hydroxytoluenes (BHT), and *dl*-α-tocopherol. An initial induction phase occurs supporting the concept that the reaction is free radical initiated in nature. The reaction is likely to proceed by the following mechanism (Roehm *et al.*, 1971a).



$$R'' \cdot + O_2 \longrightarrow ROO \cdot \longrightarrow \text{conventional peroxidation}$$

It is likely that once the alkyl free radicals occur the reaction will proceed as in spontaneous air oxidation leading to hydroperoxides and ultimately to malonaldehyde. Diene conjugation and separation of the oxidation products by thin-layer chromatography supports this mechanism. While α-tocopherol is less effective than BHA or BHT, significant antioxidant activity is present. Phenolic antioxidants probably react with the hydroperoxy free radical rather than inhibiting the initial reaction of NO_2 with PUFA.

Ozone oxidation proceeds by other means. The kinetics of O_3 oxidation of an emulsion of methyl linolenate are complex. The induction phase typical of air oxidation or of NO_2 oxidation in the presence of antioxidants is not observed. All of the antioxidants tested were of the same order of effectiveness. The production of malonaldehyde is not proportional to diene conjugation, as measured by ultraviolet absorption. Thin-layer chromatography of the products demonstrated the almost complete conversion of oleic and linoleic acids to their corresponding ozonides, peroxides, and polymers under conditions of anhydrous thin films. Emulsions contained ozonides and a diverse mixture of polar products. The O_3 oxidation probably proceeds by the direct addition of O_3 to the ethylene group.



$$RCH_2OO \cdot + {}''RH \longrightarrow \text{conventional peroxidation}$$

Direct O_3 addition results in a malonozonide (I), which spontaneously cleaves, giving rise to the Criegee zwitterion (II) and an aldehyde (III). Reaction of these products forms the ozonide (IV). Ozonides are quite stable. Their biological activity is unknown. Ozonides could be cleaved to give rise to peroxides. In aqueous emulsions the Criegee zwitterion can react with water to give the peroxy free radical (V); conventional peroxidation can then follow. These events appear to proceed at different rates, making the total reaction complex.

Phenolic antioxidants are likely to react only with the hydroperoxyl free radical (V). The O_3 oxidation is much more rapid and complete than the NO_2 oxidation. Ozone is more toxic than NO_2. The LC_{50} for the rat at 3 hr for O_3 is 21.8 ppm (Mittler *et al.*, 1954) compared to 88 ppm for NO_2 (Gray *et al.*, 1954). The differences in reaction mechanism and rate may well explain the greater toxicity of O_3.

Vitamin E Depletion and Ozone and Nitrogen Dioxide Toxicity. The erythrocyte is a sensitive and accessible indicator of the vitamin E status of animals. Prior to O_3 exposure all animals were equilibrated to the experimental diet (Table I) containing 100 mg/kg of *dl*-α-tocopherol. Half of the animals were then placed on the same diet free of tocopherol and exposed to either 0.5 ppm of O_3 or filter air. Eighty to 100% hemolysis under these conditions corresponds to a serum tocopherol <0.5 mg/100 ml. At 23 days none of the unexposed group showed hemolysis, while three-fifths of

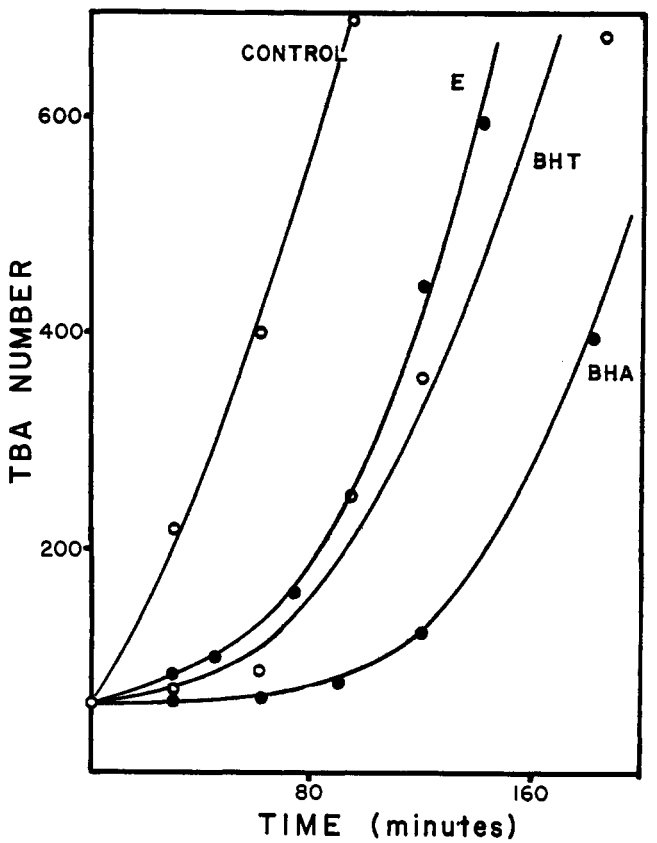


Figure 1. Oxidation of thin films of methyl linoleate in the presence of 1.5 ppm of NO_2. TBA number equals ppm of malonaldehyde

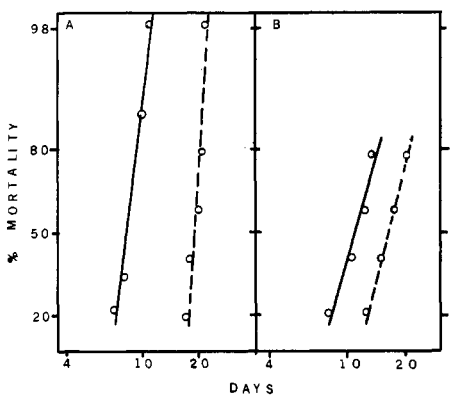


Figure 2. Mortality of vitamin E-deficient and supplemented rats exposed to NO_2 and O_3. A. O_3 exposure to 1.5 ppm. B. NO_2 exposure to 33 ppm. Solid lines represent vitamin E-deficient rats; dashed lines, supplemented rats. See text for diet composition

the exposed group did (Table II). A similar level of depletion did not occur with the unexposed group until the 36th day. Erythrocytes from animals receiving continued supplements of tocopherol did not show hemolysis, regardless of exposure. Ozone clearly accelerates the depletion of tocopherol reserves.

Tocopherol reserves also influence the ability of animals to survive lethal concentrations of both O_3 and NO_2. Figure 2A illustrates that the LT_{50} for 1.5 ppm of O_3 for animals depleted of tocopherol for 4 weeks was 8.2 days *vs.* 18.5 days for continuously supplemented animals. Similar effects occur with NO_2 (Figure 2B) but are less dramatic. The LT_{50} for depleted animals exposed to 33 ppm of NO_2 is 11.1 days *vs.* 17 days for those continuously supplemented. These data clearly demonstrate the influence of tocopherol status upon the acute toxicity of O_3 and NO_2.

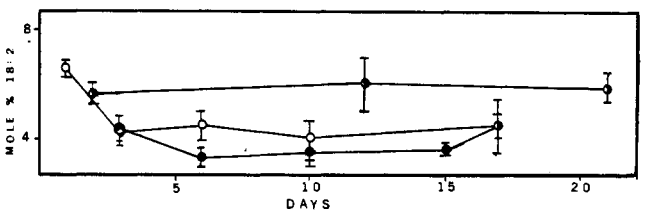


Figure 3. Linoleic acid concentration of lavage lipids from rats exposed to 1.6 ppm of O_3, 3.0 ppm of NO_2 or filtered air. Solid circles represent O_3-exposed rats; open circles represent NO_2-exposed; and half-filled circles represent air-exposed. Vertical bars represent the group standard error

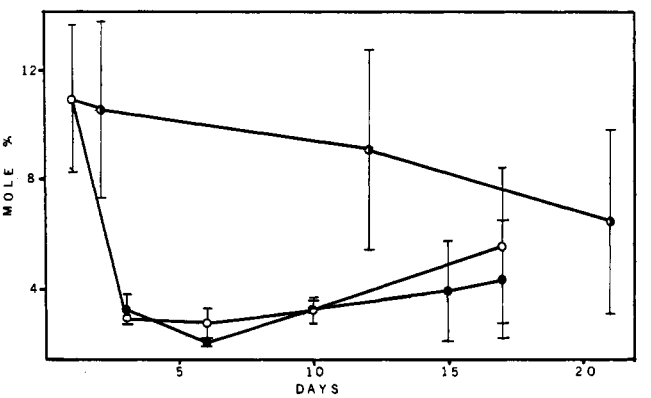


Figure 4. Linolenic acid concentration of lavage lipids from rats exposed to 1.6 ppm of O_3, 3.0 ppm of NO_2 or filtered air. Solid circles represent O_3-exposed rats; open circles represent NO_2-exposed; and half-filled circles represent air-exposed. Vertical bars represent the group standard error

Table II. Hemolysis by Dialuric Acid of Erythrocytes from Rats Exposed to 0.5 ppm of Ozone and Fed a Vitamin E-Free Diet

Group	Exposure time days[a]							
	3	7	11	16	23	29	36	45
Filtered air	0/4	0/4	0/4	0/4	0/4	1/5	2/5	4/5
0.5 ppm of ozone	0/4	0/4	0/4	0/4	3/4	4/5	5/5	5/5

[a] Values reported as number of positive samples over number of samples taken.

Oxidant Influence on Lung Lipids. The lung is the organ most exposed to these oxidants. The pathology of O_3 and NO_2 intoxication has been described as a general and nonspecific edema. Analyses of the lung must take into account the structure and function of the lung. The pressure-volume relationship of the mammalian lung is a nonlinear function. The inflation and deflation pressures are distinctly separate. While large differences exist in the size of alveoli (the gas exchange units of the lung), the unique pressure-volume relationship of the lung allows the alveoli to inflate simultaneously at very low pressures, preventing their coalescence into large structures of uniform diameter. Further, the alveoli are prevented from collapse by the higher deflation pressure. These properties appear confined to the lung lining material, which possesses great surface activity and, hence, is referred to as the lung surfactant. The surfactant is composed mostly of dipalmitoyl lecithin but contains other fatty acids in lesser amounts esterified to a variety of molecular species. The lung is divided for analysis into two functional parts: lavage or endobronchial washings indicative of the surfactant, and the remaining tissue, referred to here as simply tissue.

Acute exposure to NO_2 or O_3 causes marked changes in the total fatty acid composition of the lavage, as shown in Figures 3 and 4 for 18:2 and 18:3 without major tissue changes.

Table III. Total Fatty Acid Composition of Lung Tissue Lipids from Rats Exposed to 10 ppm of NO_2 for 4 Weeks[a]

Fatty acid[b]	Mole % (±SEM) of total lung tissue fatty acids[c]			
	Supplemented control	Deficient control	Supplemented NO_2 exposed	Deficient NO_2 exposed
16:0	25.60 ± 0.13	25.94 ± 0.64	28.27 ± 0.95	28.59 ± 0.25
16:1	4.55 ± 0.32	4.66 ± 0.16	5.43 ± 0.07	5.80 ± 0.30
18:0	10.29 ± 0.50	10.03 ± 0.13	10.03 ± 0.13	9.57 ± 0.54
18:1	24.56 ± 0.66	24.25 ± 1.43	25.10 ± 0.05	28.62 ± 1.36
18:2	7.86 ± 0.27	7.85 ± 0.16	6.61 ± 0.36	6.43 ± 0.13
18:3	6.02 ± 1.02	6.67 ± 0.45	4.81 ± 0.55	4.89 ± 0.53
20:4	5.57 ± 0.16	4.90 ± 0.43	5.14 ± 0.17	4.58 ± 0.88
20:5	3.07 ± 0.34	3.28 ± 0.24	3.06 ± 0.08	2.15 ± 0.24
22:5	4.61 ± 0.57	4.69 ± 0.43	3.79 ± 0.09	2.78 ± 0.42
22:6	1.17 ± 0.20	1.47 ± 0.08	1.16 ± 0.13	1.04 ± 0.12

[a] Minor components omitted for clarity. [b] The notation for fatty acids is X:Y, where X is the number of carbon atoms and Y, the number of unsaturations. [c] N = 10.

Table IV. Total Fatty Acid Composition of Lung Tissue Lipids from Rats Exposed to 1.0 ppm of O_3 for 9 Days[a]

Fatty acid[b]	Mole % (±SEM) of total lung tissue fatty acids[c]			
	Supplemented control	Deficient control	Supplemented O_3 exposed	Deficient O_3 exposed
14:0	1.07 ± 0.11	1.08 ± 0.11	1.03 ± 0.11	0.83 ± 0.04
16:0	24.68 ± 0.81	25.16 ± 0.34	27.62 ± 0.65	29.22 ± 0.84
16:1	4.97 ± 0.49	4.85 ± 0.33	5.22 ± 0.67	4.34 ± 0.20
18:0	9.73 ± 0.67	9.72 ± 0.63	9.76 ± 0.83	9.73 ± 0.37
18:1	24.09 ± 1.87	23.85 ± 2.58	23.42 ± 2.99	20.70 ± 0.47
18:2	7.65 ± 0.45	7.55 ± 0.47	6.97 ± 0.47	6.14 ± 0.74
18:3	6.19 ± 0.67	6.14 ± 0.98	4.98 ± 1.06	3.43 ± 0.21
20:4	6.01 ± 0.59	6.01 ± 0.90	7.18 ± 0.93	8.87 ± 0.81
20:5	3.25 ± 0.68	3.67 ± 0.51	3.27 ± 0.86	2.21 ± 0.16
22:5	5.35 ± 1.02	4.77 ± 0.75	4.12 ± 1.26	5.80 ± 1.67
22:6	1.67 ± 0.10	2.05 ± 0.54	1.64 ± 0.23	3.50 ± 0.43

[a] Minor components omitted for clarity. [b] The notation for fatty acids is X:Y, where X is the number of carbon atoms and Y the number of unsaturations. [c] Number of animals in each group was six.

Table V. The Influence of Ozone Exposure at 0.5 ppm for 65 Days on Sulfhydryl Content of Rat Lung Tissue

	Vitamin E-supplemented rats		Vitamin E-deficient rats	
	Air exposed[a]	Ozone exposed[a]	Air exposed[a]	Ozone exposed[a]
Body wt	379 ± 24.0	373 ± 36.7	374 ± 17.6	368 ± 48.0
Lung % body wt	0.41 ± 0.02	0.48 ± 0.07	0.41 ± 0.04	0.48 ± 0.08
% Moisture	76.2 ± 3.4	76.0 ± 1.0	76.6 ± 1.4	76.4 ± 1.2
Total SH (μg/g of dry tissue)	35.8 ± 4.8	31.3 ± 4.0	31.3 ± 3.7	29.0 ± 3.5

[a] Values represent mean ± standard error for seven animals.

Tocopherol-depleted animals exhibit losses of lavage unsaturated fatty acids consonant with an oxidative hypothesis. Breathing filtered air during depletion does not prevent the general decline in these fatty acids, indicating that spontaneous air oxidation still occurs in depleted animals.

The human experience is more chronic than acute and consequently we have chosen to focus upon the tissue changes in fatty acids to test our hypothesis of chronic toxicity. Alteration of lung fatty acids by diet is slow, and the lavage fatty acid composition is maintained at a relatively constant composition (Menzel, 1971). Lavage fatty acids are maintained at the expense of tissue fatty acids under either dietary or environmental stress.

Table III shows the total fatty acid composition of lung lipids from animals exposed for 4 weeks to 10 ppm of NO_2. The kinetics and end products of NO_2 oxidation are very similar to that of spontaneous oxidation. Consequently, the changes induced by tocopherol deficiency alone should be accentuated by NO_2 exposure. The tocopherol-supplemented control group contained a significantly lower concentration of 18:2 and 18:3 than did the deficient group. Four weeks of exposure produced depletion of PUFA similar to those reported by Witting (1967) for tocopherol depletion of muscle phospholipids. Tocopherol-deficient rats showed significant ($p > 0.05$) reductions in 18:2, 18:3, 20:5, 22:5, and 22:5 with corresponding increases in 18:0, 16:1, and 18:1. No changes in 20:4 were observed. Tocopherol-supplemented animals exposed to NO_2 had reduced levels of 18:2, 18:3, and 22:6, but these levels were not significant at the 5% level of probability. A comparison of the two exposed groups shows 20:5 and 22:6 levels significantly reduced in the deficient group. If NO_2 increases the oxidation of PUFA *in vivo* by a similar mechanism to spontaneous oxidation, then selective oxidation of higher PUFA should occur. These and other experiments suggest that NO_2 accelerates the oxidation of PUFA as hypothesized.

Ozone, on the other hand, presents a more complex picture. In aqueous systems, antioxidants were only partially effective in preventing oxidation. Ozone also accelerates the depletion of tocopherol stores. Table V illustrates the tissue fatty acid composition after 9 days of exposure to 1.0 ppm of O_3 or at the LT_{50} for tocopherol-deficient rats. Note the decreased levels of 18:1 and 18:3. The most striking change is the increase in 20:4. The content of the highly peroxidizable

22:6 also increased. Longer term exposure at lower levels of 0.5 ppm of O_3 clearly indicated these changes. These trends are more clearly shown in a plot of the time course of alteration of tissue fatty acid (Figure 5). Oleic (18:1) and palmitoleic (16:1) acid concentrations fell, while the content of 18:0 and 16:0 increased concomitantly. The most notable change, however, was a marked rise in arachidonic acid (20:4).

These data suggest that the critical factor may not be oxidation, as much as derangement of fatty acid biosynthesis. Witting (1967) has observed similar effects, including an increased incorporation of acetate into PUFA, even though PUFA *in toto* was reduced presumably by oxidation. Bernhard *et al.* (1963) have observed a specific increase in arachidonic acid biosynthesis and content of liver lipids during tocopherol depletion. They have suggested that the increased arachidonic acid synthesis may be due to the lack of available tocopherol for the regulation of the oxidative elongation and desaturation steps. The alterations in fatty acid composition occurred most rapidly at those times when lung edema and death through respiratory failure was observed. With this diet, levels of arachidonic acid in excess of 8.5% were associated with edema.

Oxidation of Thiols. We have dwelt considerably upon fatty acid composition and its relationship to tocopherol as an indicator of lipid oxidation, but we must report effects upon other components which may be equally involved in these effects. Previously, Menzel has shown that O_3 very readily oxidizes NADH and NADPH, but that they are still enzymatically reducible (Menzel, 1971b). Thiols, on the other hand, appear oxidized to higher oxidation states than the disulfide. Glutathione, in particular, is easily oxidized and a significant portion cannot be reduced by NADPH–glutathione reductase. Accordingly, are the changes induced by tocopherol depletion or oxidant exposure related to the general redox state of the lung? Figure 6 illustrates the serum reduced glutathione levels which fell on O_3 exposure, regardless of tocopherol status. The tissue sulfhydryl content as shown in Table V was unaffected, despite the 25% reduction in serum reduced glutathione levels.

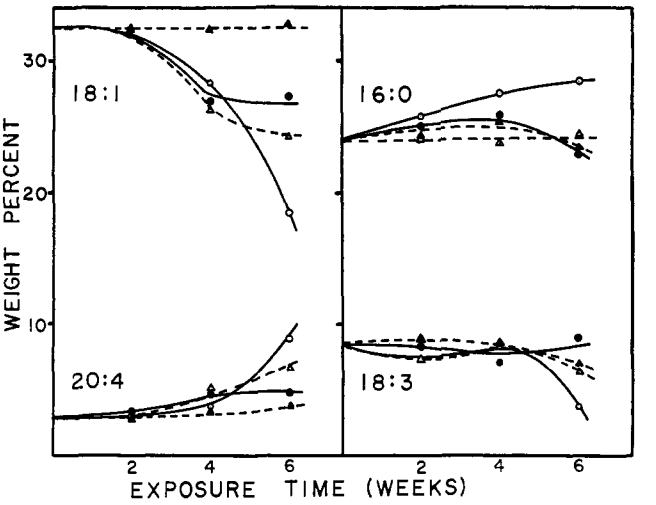


Figure 5. Effect of long-term O_3 exposure on rat lung total lipids. Air-exposed, vitamin E-supplemented -▲--▲-; air-exposed, vitamin E-deficient -●—●-; O_3-exposed, vitamin E-supplemented -△-△-; O_3-exposed, vitamin E-deficient -○—○-

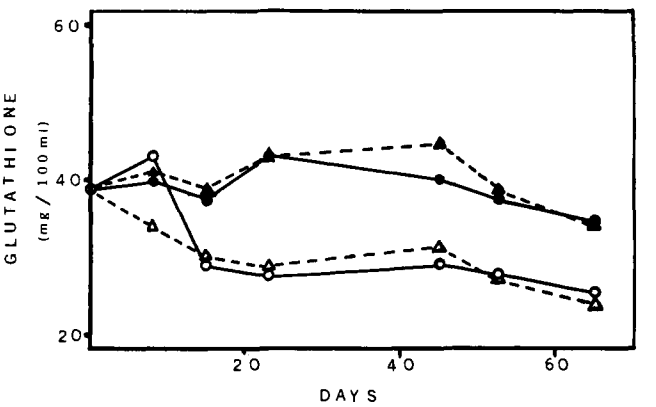


Figure 6. Serum reduced glutathione response to O_3 exposure. Air-exposed, vitamin E-supplemented -▲--▲-; air-exposed, vitamin E-deficient -●—●-; O_3-exposed, vitamin E-supplemented -△-△-; O_3-exposed, vitamin E-deficient -○—○-

DISCUSSION

PUFA are readily oxidized by NO_2 and O_3, and the oxidation is partially retarded by phenolic antioxidants. While similarities exist, there are differences in the composition of the final reaction products and the reaction mechanism (as hypothesized) between conventional PUFA peroxidation and NO_2 and O_3 oxidation. Hydroperoxides are formed in variable amounts; therefore, it is better to refer to this as an "oxidation" rather than "peroxidation," especially snce hydroperoxidases have not been found *in vivo* as yet.

The evidence presented for this reaction *in vivo* is indirect. Both NO_2 and O_3 acute toxicity are a function of the tocopherol intake and stores. Similarly, tocopherol is more rapidly depleted on exposure to oxidants. Lung tissue fatty acids are altered in their composition both by changes in tocopherol intake and by oxidant exposure. The fatty acid changes observed are primarily in those fatty acids biosynthesized by desaturation or by desaturation followed by elongation. We have attempted to maintain the lung fatty acid composition at a constant but high level of unsaturation by provision of a constant supply of dietary fatty acids. The changes in tissue fatty acid could represent the balance between alterations in rate of biosynthesis (which we have not investigated) and alterations in the rate of oxidation and hence removal.

The desaturase enzymes are of two groups: steric–oleic and linoleic–arachidonic and linolenic–docosahexenoic systems. The steric–oleic desaturase is inductive in nature and regulated by insulin and other hormones (Struijk and Beerthius, 1966; Mercuri *et al.*, 1966). Both enzyme systems are microsomal and may require lipid cofactors. McCay *et al.* (1971) have suggested that α-tocopherol functions to stabilize the microsomal membrane from free radical attack during TPNH-dependent oxidations such as desaturation. The steric–oleic desaturase could be more susceptible to degradation and hence result in the decline in oleic acid.

Arachidonic acid appears to be the key PUFA. O_3 oxidation appears to increase the arachidonic acid content, suggesting a more active desaturase. The relative rates of oxidation proposed by Witting and Horwitt (1964) and by Witting (1967) are based on the oxidation *in vitro* of pure PUFA. Structural associations within cell membranes may well alter the relative rates of oxidation *in vivo*. Arachidonic acid is almost the exclusive source of malonaldehyde in the oxidation of microsomes by peroxidation (Niehaus and Samuelson, 1968). Arachidonic acid increases have also been reported as a result

of tocopherol deficiency in liver (Bernhard *et al.*, 1963) and in muscle (Witting, 1967). Arachidonic acid, because of its essentiality to the function of cellular membranes, may be closely associated with enzymatically active sites or transport areas and hence be particularly prone to oxidation by both internal and external free radical sources.

Lastly, one should recognize that tocopherol deficiency augmented by oxidant exposure is likely to present multiple foci, in the classical sense. Alterations simultaneously in lipid, protein, sulfhydryl compounds, and nucleotide oxidation states are likely. Serum-reduced glutathione levels are depressed by oxidant exposure but are unaffected by tocopherol intake. Potential interactions may occur since many reactions require sulfhydryl compounds (acyl CoA in desaturases) and since cystine and methionine have been shown to reverse the creatinuria of tocopherol deficiency without alteration of the lipid peroxidation (Desai *et al.*, 1964). In any event, the complexity and, unfortunately, the catastrophic nature of tocopherol deficiency may well preclude human experimentation for some time, but our data suggest that it might be well to recommend the provision of adequate levels of tocopherol for the inhabitants of air-polluted areas.

ACKNOWLEDGMENT

We wish to thank R. M. Danner and J. G. Hadley for their technical assistance. This work was supported in part by P.H.S. Contract 22-68-61.

LITERATURE CITED

Ackman, R. G., Sipos, J. C., *J. Amer. Oil Chem. Soc.* **41,** 377 (1964).
Bernhard, K., Leisinger, S., Pedersen, W., *Helv. Chim. Acta* **46,** 1767 (1963).
Bonner, W. A., *J. Chem. Educ.* **30,** 452 (1953).
Buetler, E., Duron, O., Kelley, B. M., *J. Lab. Clin. Med.* **61,** 882 (1963).
Dam, H., Glavind, J., *Nature (London)* **142,** 1077 (1938).
Desai, I. D., Calvert, C. C., Scott, M. L., *Arch. Biochem. Biophys.* **108,** 60 (1964).
Draper, H. H., Goodyear, S., Barbee, K. D., Johnson, B. C., *Brit. J. Nutr.* **12,** 89 (1958).
Draper, H. H., Csallany, A. S., *Proc. Soc. Exptl. Biol. Med.* **99,** 739 (1959).
Draper, H. H., *Proc. Soc. Exptl. Biol. Med.* **102,** 737 (1959).
Evans, H. M., Bishop, K. S., *Science* **56,** 650 (1922).
Folch, J., Lees, M., Stanley, G. H. S., *J. Biol. Chem.* **226,** 497 (1957).
Friedman, L., Weiss, W., Wherry, F., Kline, O. L., *J. Nutr.* **65,** 143 (1958).
Goldstein, D. B., Buckley, R. D., Cardenas, R., Balchum, O. J., *Science* **169,** 605 (1970).
Gray, E. L., Goldberg, S. B., Patton, F. M., *Arch. Ind. Hyg. Occ. Med.* **10,** 423 (1954).
Matill, H. A., Golumbic, C., *J. Nutr.* **23,** 625 (1942).
McCay, P. B., Poyer, J. C., Pfeifer, P. M., May, H. E., *Lipids* **6,** 297 (1971).
Menzel, D. B., *Ann. Rev. Pharmacol.* **10,** 379 (1970).
Menzel, D. B., *Arch. Environ. Health* **23,** 149 (1971a).
Menzel, D. B., unpublished data, 1971b.
Mercuri, O., Peluffo, R. O., Bremmer, R. R., *Biochim. Biophys. Acta* **116,** 409 (1966)
Mittler, S., Hedrick, D., King, M., Gaynor, A., *Ind. Med. Surg.* **25,** 301 (1954).
Niehaus, W. G., Jr., Samuelson, B., *Eur. J. Biochem.* **6,** 126 (1968).
Roehm, J. N., Hadley, J. G., Menzel, D. B., *Arch. Environ. Health* **23,** 142 (1971a).
Roehm, J. N., Hadley, J. G., Menzel, D. B., *Arch. Intern. Med.* **128,** 88 (1971b).
Saltzman, B. E., Gilbert, N., *Ann. Chem.* **31,** 1914 (1959).
Schwarz, K., *Z. Physiol. Chem.* **281,** 109 (1944).
Schwarz, K., Bieri, J. G., Briggs, G. M., Scott, M. L., *Proc. Soc. Exp. Biol. Med.* **95,** 621 (1957).
Sedlak, J., Lindsay, R. H., *Anal. Biochem.* **25,** 192 (1968).
Shaw, A. M., Menzel, D. B., Brooksby, G. A., Leon, H. A., *J. Nutr.* submitted for publication (1972).
Struijk, C. B., Beerthius, R. K., *Biochim. Biophys. Acta* **116,** 12 (1966).
Witting, L. A., Horwitt, M. K., *J. Nutr.* **82,** 19 (1964).
Witting, L. A., *Lipids* **2,** 109 (1967).

Received for review July 9, 1971. Accepted March 13, 1972. Presented at symposium on Chemical Aspects of Nutrition Needs, Division of Agricultural and Food Chemistry, 161st ACS Meeting, Los Angeles, Calif., March–April 1971.

Protective Effect of Vitamin E on Plasma Lipid Dienes in Man

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Since previous studies indicated that lipid peroxidation may be the molecular basis of experimental ethanol-induced hepatic injury, studies were undertaken to determine if diene conjugation could be detected in plasma lipids in conditions of acute and chronic alcoholism. Plasma lipids of human subjects, both normal and diseased, revealed the presence of conjugated diene absorption patterns. The presence of conjugated dienes in plasma lipids, particularly in the phospholipid fraction, may well result from *in vivo* peroxidative events since the administration of lipid antioxidant, as mixed tocopherols, was associated with a significant reduction in plasma lipid conjugated diene levels. This reduction was associated with a significant enhancement in plasma lipid soluble antioxidant activity. Conversely, removal of supplemental vitamin E was associated with a fall in plasma lipid antioxidant activity and a rise in conjugated diene levels, suggesting that the presence of the abnormal conjugated dienes in plasma lipids might be due to a relative antioxidant deficient state or antioxidant imbalance related to excessive polyunsaturated fat intake.

Previous studies from this laboratory have led to the hypothesis that the mechanism of hepatic cell injury, after the administration of such agents as ethanol or carbon tetrachloride, was possibly due to an enhanced peroxidation of lipids (Di Luzio, 1964; Di Luzio and Costales, 1965; Di Luzio, 1966; Comporti *et al.*, 1967; Di Luzio, 1967; Di Luzio and Poggi, 1967; Di Luzio and Hartman, 1967; Di Luzio and Hartman, 1969b). The induced lipid peroxidation was postulated to be related to an ethanol-induced free radical attack on unsaturated lipids of specific hepatic subcellular organelles due to a decreased lipid soluble antioxidant level (Di Luzio and Hartman, 1969a). The protection of animals from the acute or chronic effects of such hepatotoxic agents as ethanol and carbon tetrachloride by the administration of antioxidants was proposed to be caused by an inhibition of lipid peroxidation (Di Luzio, 1964; Di Luzio and Costales, 1965; Di Luzio, 1966; Comporti *et al.*, 1967; Di Luzio, 1967;

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