EFFECT OF TOCOPHEROL SUPPLEMENTATION ON NITROGEN DIOXIDE TOXICOSIS IN RATS

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ABSTRACT

EFFECT OF TOCOPHEROL SUPPLEMENTATION ON

NITROGEN DIOXIDE TOXICOSIS IN RATS

By

James Daniel McKean

The objective of this research was to determine the effects of vitamin E supplementation on chronic nitrogen dioxide (NO_2) exposure in the weanling rat. A torula yeast based diet was fed to all test animals to deplete hepatic tocopherol stores. Tocopherol supplementation was 100 I.U./rat of alpha-tocopherol, subcutaneously, per week. Two nitrogen dioxide levels (20 and 30 ppm), administered 24 hours/day until all rats in their respective treatment groups had died, were used to ascertain the possibility of a graded response.

Rats exposed to NO_2 exhibited signs of nasal, ocular and respiratory irritation before death. Rats not exposed to NO_2 were normal. Tocopherol supplementation had no measurable effect on clinical signs or on time of death.

Macroscopic changes including pulmonary edema, congestion and hemorrhages were more severe in the 30 ppm exposure groups. Lesions in non-exposed rats were mild hepatic congestion.

Microscopic changes in the unsupplemented diet control group were limited to congestion of hepatic lobules and slight centrolobular hepatic necrosis. Pulmonary changes in rats exposed to 30 ppm NO_2 consisted of perivascular, peribronchiolar, and alveolar edema, vascular

congestion, loss of ciliated bronchiolar epithelium and mild bronchiolitis. Bronchiolar proliferation, bronchiolitis, mild perivascular and peribronchiolar edema, emphysema, alveolar wall thickening, and pneumonia of variable severity were seen in the 20 ppm NO_2 group.

Growth rate was markedly depressed by NO_2 exposure. Some depression of appetite was observed in tocopherol supplemented rats. Nitrogen dioxide had no measurable effect on hepatic tocopherol levels in the supplemented or unsupplemented groups. At 20 ppm NO_2, rats unsupplemented with tocopherol had heavier lungs than their treated counterparts. It was concluded that tocopherol supplementation had no effect on NO_2 toxicosis at 20 and 30 ppm levels.

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INTRODUCTION

Evans and Bishop (1922) discovered a dietary factor essential for reproduction in rats which was later isolated and designated tocopherol. Since its discovery, numerous syndromes, in addition to infertility in rats, have been attributed to vitamin E deficiency.

Many investigators have attempted to explain the activity of vitamin E through its ability to prevent oxidation of polyunsaturated fatty acids (PUFA). Numerous "stressing" substances were used to accelerate the production of hepatic necrosis, one of the manifestations of vitamin E deficiency in rats. Among these were PUFA, silver acetate, iron dextran, carbon tetrachloride, and ethanol. Many investigators related the toxic effects of these substances to cellular membrane lipid peroxidation. Protection of membranes by vitamin E was advanced as proof of the antioxidant theory.

Exposures to nitrogen dioxide, ozone, and other oxidant gases associated with photochemical smog produced pulmonary cellular membrane lipid peroxidation in rats. An ability of vitamin E to protect cellular membranes from lipid peroxidation and the resulting insidious changes of fibrosis and emphysema were observed in chronic low-level exposures to these gases.

The literature reviewed here will primarily concern low-level exposure to nitrogen dioxide and its possible interaction with vitamin E metabolism. A consideration of acute industrial and agricultural poisonings due to nitrogen dioxide was presented by Giddens (1966).

The internal combustion engine, as a source of nitrogen oxides (Stokinger, 1964), added another dimension to nitrogen oxide toxicosis. Rather than the acute, high-level exposures to nitrogen oxides observed in industry and agriculture, this syndrome was characterized by chronic, low-level concentrations and insidious changes. The most obvious manifestation of the oxidant substances in the atmosphere, "photochemical smog", has occurred with increasing frequency in many urban areas.

OBJECTIVES

The objective of this research was to determine the effect of vitamin E on chronic nitrogen dioxide exposure in the weanling rat.

Specific aims were:

1. To determine the effect of tocopherol supplementation on nitrogen dioxide toxicosis.

2. To characterize the histopathologic changes observed in nitrogen dioxide toxicosis and the possible protective action of vitamin E.

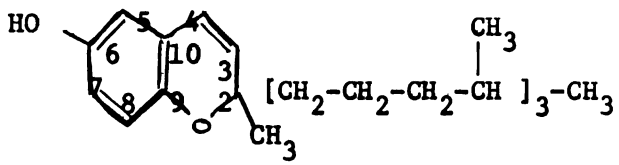
3. To determine the effects of vitamin E deficiency on survival times of rats exposed to 20 ppm nitrogen dioxide.

4. To utilize the information obtained to re-evaluate nutritional requirements of man and animals living in areas of high oxidant gas concentrations.

LITERATURE REVIEW

Chemistry of Tocopherols

Vitamin E was initially isolated from wheat germ oil by Evans *et al.* (1936) and has since been found in large quantities in many plant oils and lipids. Later, the configuration of vitamin E was reported to be not a single structure, but a group of derivatives of tocol, 2-methyl-2-trimethyl-tridectyl, 6-hydrochromane. Four of the derivatives were found in nature and collectively called tocopherols (Figure 1).



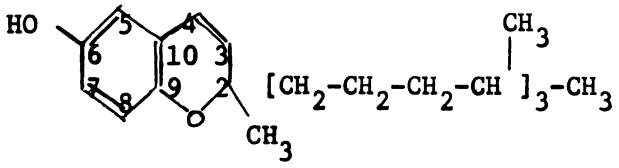
Tocol

Alpha tocopherol	5,7,8, Trimethyltocol
Beta tocopherol	5,8, Dimethyltocol
Gamma tocopherol	7,8, Dimethyltocol
Delta tocopherol	8, Methyltocol

Figure 1. Nomenclature and chemical structures of tocopherol.

Pennock *et al.* (1964) reported that in addition to the 4 tocopherols, 4 tocotrienol derivatives existed in nature (Figure 2).

Of the natural occurring derivatives, alpha-tocopherol is the most widely distributed and the most biologically active. The reason for the greater potency of alpha-tocopherol was not clearly defined, although



Tocotrienol

Alpha-tocotrienol	5,7,8, Trimethyltocotrienol
Beta-tocotrienol	5,8, Dimethyltocotrienol
Gamma-tocotrienol	7,8, Dimethyltocotrienol
Delta-tocotrienol	8, Methyltocotrienol

Figure 2. Nomenclature and chemical structures of tocotrienol.

some researchers proposed that the greater methylation of the benzene ring increased its antioxidant abilities.

Biochemical Activity of Vitamin E

Scott (1969) extensively reviewed the vitamin E deficiency syndromes of domestic and laboratory animal species and the relationships of tocopherols, polyunsaturated fatty acids (PUFA), selenium, sulfur amino acids, and synthetic antioxidants to the development of these syndromes. The diversity of manifestations and the complexity of interactions has concerned investigators for many years. The absence of a single chemical or metabolic aberration to explain the wide spectrum of lesions fostered numerous theories of tocopherol activity. Three of these theories are: (1) a coenzymatic activity in cellular respiration, (2) a protection of cellular and subcellular elements from oxidation, and (3) an increased cellular membrane stability through an unknown mechanism.

Schwarz (1965) and others have postulated that tocopherol and selenium act as specific coenzymes in essential metabolic pathways. At present, several sites of coenzymatic activity have been advanced.

Schwarz observed respiratory declines from liver slices and homogenates of deficient animals and proposed that the activity of lipoyl dehydrogenase in the alpha-ketogluturate oxidase system was sensitive to vitamin E or selenium deficiency. Nason and Lehman (1966), using semipurified rat skeletal muscle preparations, observed increased NADH oxidation as a result of increased cytochrome C reductase activity. Porter and Fitch (1966) observed an anemia in vitamin E deficient monkeys which was traced to a disruption of delta aminolevulinic acid synthesis in bone marrow. Remission of the anemia followed vitamin E supplementation. A similar response was seen in vitamin E-deficient rabbits. A direct action in the enzyme systems was proposed as the main function of vitamin E.

The *in vitro* antioxidant activity of tocopherols was known several years prior to Davis and Moore's (1941) proposal of *in vivo* activity. They observed a decreased oxidation of hepatic vitamin A stores in rats supplemented with vitamin E. Many *in vitro* and *in vivo* experiments utilized tocopherol analogs and synthetic antioxidants to spare or replace alpha-tocopherol. These studies illustrated various abilities to protect tissues from signs of vitamin E deficiency. Ethoxyquin, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and methylene blue were among the compounds used. N,N-diphenyl p-phenyl diamine (DPPD) was reported to be the most active synthetic antioxidant and to prevent the majority of vitamin E deficiency signs including fetal resorption in rats (Green and Bunyan, 1969). These compounds were structurally dissimilar to vitamin E, but all possessed the ability to donate an electron readily. The stoichiometric differences of these compounds was associated with their absorption, tissue retention, and excretion and, therefore, with the observed differences in ability to prevent vitamin E deficiency lesions.

The functions of biological antioxidants were reported to consist of free radical scavengering, reduction of free radical chain reactions, reduction of electron mobility, and trace metal chelation. A physiologic equilibrium between the oxidant and antioxidant substances in the cell was proposed. The disturbance of this balance was offered as a cause of cellular damage (Hess and Menzel, 1971). The formation of lipoperoxides by the oxidation of PUFA, a normal physiologic event, increased the concentration of oxidant substances. In the absence of electron donors, the free radicals produced acceleration of the PUFA oxidation. An increased oxidant-antioxidant ratio and the resultant increased lipoperoxide production were postulated to cause damage to proteins, enzyme systems, vitamins, and lipid components of cellular membranes (Roubal, 1964). The peroxyl intermediates, which increased as a relative antioxidant deficiency occurred, were proposed to cause this damage. Aldehydes and other lipid oxidation metabolites could not, initially, be eliminated from consideration. However, Roubal (1971) utilized electron paramagnetic resonance to detect free radical activity and reported that free radicals were the source of cellular damage. Desai and Tappel (1963) reported that the damage to proteins and amino acids by peroxidation was of the same type and magnitude as that of ionizing radiation.

Green and Bunyan (1969) assembled a critique of the antioxidant theory and its relationship to PUFA, selenium, sulfhydryl compounds, and various hepatic "stress" substances. They expressed the opinion that evidence supporting the antioxidant theory was circumstantial. The inability of synthetic antioxidants to completely protect animals from signs of vitamin E deficiency was presented as a major stumbling block. The technical aspects of malonaldehyde (MDA) measurements, a test used

extensively for indirect quantitation of tissue lipid peroxidation, were examined and discrepancies in interpretation were illuminated. Barber (1963) proposed that MDA production *in vitro* estimated the balance of oxidant-antioxidant components and not the peroxidation of lipids. With vitamin E deficiency, MDA values were consistently elevated when compared to controls. Horwitt (1965) speculated that changes in the oxidant-antioxidant balance were buffered by many reducing substances including vitamin E. He stated that imbalance could result in insidious cellular changes.

Lucy and Dingle (1964) reported a possible stabilizing effect of vitamins A and E on biological membranes. Seward *et al.* (1964) proposed that vitamin E affected membrane permeability in a manner similar to vitamin A. Both groups postulated that vitamin E was incorporated into the membranes along with lipids and proteins. This was considered an important part of its activity. Whether the vitamin E activity was the result of an antioxidant function, or another unknown mechanism, was not elucidated.

Pathologic Changes of Vitamin E Deficiency

The manifestations of vitamin E deficiency were observed to be encephalomalacia (Pappenheimer and Goetsch, 1931) and exudative diathesis in chickens (Dam and Glavind, 1938); hepatic necrosis and muscular lesions in swine (Obel, 1953); myopathy in herbivores (Blaxter *et al.*, 1951); hepatic necrosis, renal tubular degeneration, testicular degeneration, incisor depigmentation, erythrocyte fragility, fetal resorption and muscular degeneration in rats. Fetal resorption in rats (Emerson and Evans, 1939) was used for many years as a biological assay of the activity in tocopherol analogs and synthetic antioxidants. The only

antioxidant to simulate alpha-tocopherol activity with this technique was DPPD (Green and Bunyan, 1969). Erythrocyte fragility was proposed as an *in vitro* assay of vitamin E deficiency in man and animals (Rose and Gyorgy, 1950). Dialuric acid, hydrogen peroxide, other oxidizing agents, and hypotonic solutions of saline were used to assess erythrocyte fragility (Hawk's Physiologic Chemistry, 1965). Alpha-tocopherol deficiency increased the susceptibility of erythrocytes to hemolysis while addition of alpha-tocopherol inhibited this change (Rose and Gyorgy, 1950).

Hepatic necrosis in rats, a vitamin E and selenium responsive disorder, was produced by several investigators, with many hepatic stress substances (Green and Bunyan, 1969). The necrosis was attributed to increased lipid peroxidation resulting from antioxidant deficiency. Although the biochemical aberrations which caused hepatic necrosis were not clearly defined, Schwarz (1965) described 3 distinct developmental phases: (1) an induction phase, (2) a latent phase where metabolic but no microscopic lesions were observed, and (3) a terminal phase where metabolic aberrations produced microscopic and macroscopic changes. Porta *et al.* (1968) reported a similar time sequence and by electron microscopy observed loss of microvilli, swelling, rupture, and complete disintegration of hepatic cellular membranes lining the sinusoids during the latent phase. These changes were increased as animals were maintained on the deficient diet and resulted in elevated lactic dehydrogenase (LDH) and serum glutamic oxaloacetic transaminase (SGOT) values. Machado *et al.* (1971) reported a similar ultrastructural and microscopic sequence in addition to increased lipid peroxidation at the time of membrane damage. Whether the observed rise in lipid peroxidation was a cause or an effect of the membrane instability was not critically analyzed by the

authors. Emphasis was placed on the importance of membrane instability and concurrent cytoplasmic changes. Inactivation of (Na^+-K^+)ATPase at the cellular membrane, as indicated by reduced histochemical activity in the affected membrane, was proposed as the principal cause of membrane damage. The authors speculated that (Na^+-K^+)ATPase inactivation might reverse membrane ion exchange and lead to the observed hepatocytic swelling. An alternative pathway to membrane damage might be a depression of the mitochondrial electron transport system leading to cellular respiratory decline.

Chemistry of Nitrogen Oxides

Nitrogen oxide toxicosis was extensively reviewed by Gray *et al.* (1959) and Stokinger (1964). Gray *et al.* (1959) enumerated the atmospheric oxides of nitrogen and their public health significance as follows:

Nitrous oxide	N_2O
Nitric oxide	NO
Nitrogen dioxide	NO_2
Nitrogen trioxide	N_2O_3
Nitrogen tetroxide	N_2O_4
Nitrogen pentoxide	N_2O_5

Nitrous oxide, used commonly as a surgical analgesic, required concentrations greater than 90% in air to produce lethal effects. Nitric oxide was estimated to be approximately 20% as active as nitrogen dioxide. However, nitric oxide reacted with oxygen to produce nitrogen dioxide. Pitts (1970) reported this reaction was greatly accelerated by solar energy. Nitrogen trioxide was an unstable compound which spontaneously deteriorated to nitric oxide and nitrogen dioxide. Nitrogen tetroxide, the dimer of nitrogen dioxide, was in equilibrium with nitrogen dioxide.

Nitrogen pentoxide was unstable in the presence of oxygen, yielding nitrogen dioxide and nitrogen tetroxide. Ozone was found to increase stability of this oxide in the atmosphere. The end point atmospheric oxides of nitrogen were nitric oxide, nitrogen dioxide, and nitrogen pentoxide. From the toxicologic viewpoint, nitrogen dioxide was considered the most significant. The molecular weights and boiling points of nitric oxide, nitrogen dioxide, and nitroten pentoxide were 30.01 and -151.80, 46.01 and 21.10, and 108.01 and 47 C., respectively (Handbook of Physics and Chemistry, 1965-66). Darke and Warrack (1958) reported the ratio of nitrogen dioxide/nitrogen tetroxide was temperature dependent. Theimes and Haley (1964) reported ratios of 0/100, 30/70, and 100/0 at 22, 37, and 140 C., respectively.

Haagan-Smit *et al.* (1959) reported the presence of nitrogen dioxide in cigarette, cigar, and pipe smoke. The 145-600 ppm range for cigarettes and the 1100+ ppm range for cigar and pipe smoke was markedly higher than the maximum allowable concentration (5 ppm) for industrial environments (Threshold Limit Values, 1962).

Photochemical Smog

Stokinger (1964) incriminated the internal combustion engine as the most widespread source of nitrogen oxides and estimated that 200-300 tons of nitrogen oxides were emitted yearly in Los Angeles alone. The Los Angeles County Air Pollution Control District (1967) reported that of the 920 tons of nitrogen oxides produced in Los Angeles County in 1966, 53.6% were emitted by internal combustion engines. Pitts (1970) reported the highest nitrogen oxide concentrations in Los Angeles County since 1955 was 3.9 ppm (date not given). Since the imposition of automobile emission standards in 1966, carbon monoxide and hydrocarbon

concentrations declined, but nitrogen oxide emissions substantially increased. The significance of nitrogen oxide in the production of photochemical smog by interaction with solar energy, ozone, and hydrocarbons was reported by Cvetanovic (1965) and Tabershaw *et al.* (1968). Pitts (1970) stated nitrogen oxides were emitted primarily as nitric oxide. However, solar energy activated the following reaction: $2NO + O_2 \rightarrow 2NO_2$. Tuesday (1963) proposed the interaction of nitrogen dioxide and olefins to produce free radicals as follows:

$NO_2 + h\nu \rightarrow NO + O\cdot$

olefin(R) + $O\cdot \rightarrow$ R· + products

$R\cdot + O_2 \rightarrow ROO\cdot$

$ROO\cdot + O_2 \rightarrow O_3 + RO\cdot$

$RO\cdot + ? \rightarrow$ R· + products

This series of reactions increased ozone, free radicals, and peroxyacetal nitrate (PAN) concentrations. All these compounds were established as oxidant substances. The mode of action of nitrogen oxides, ozone, free radicals, and PAN was not elucidated. Ramazzotto *et al.* (1971) reported decreased cytochrome oxidase and succinic dehydrogenase in rat tissue homogenates following nitrogen dioxide exposure. Buckley and Balchum (1965) reported depressed cellular respiration and increased aldolase and lactic dehydrogenase values following exposure. Brinkman and Lambert (1958) observed that cellular damage following ozone exposure paralleled that following experimental irradiation. The production of free radicals by irradiation and oxidant gases was proposed as the method of tissue damage.

A second theory of tissue damage was that the gases stimulated a severe inflammatory reaction which produced the lesions. Whether the

free radicals, or the irritation, or both, were responsible for the lesions was not determined.

Pathologic Changes of Nitrogen Dioxide Toxicosis

Nitrogen dioxide toxicosis was studied in numerous laboratory species. Initially, acute responses to high concentrations were examined. Hine *et al.* (1970) determined that concentrations above 50 ppm were fatal to rats and mice during a single 8-hour exposure. Fatalities were due to respiratory failure associated with pulmonary edema, laryngeal edema, venous congestion of bronchial vessels, and bronchiolitis. Edema occurred following destruction of the semi-permeability of capillary membranes. Similar inflammatory responses were observed in dogs, guinea pigs, and rabbits. However, the concentration of gas necessary to produce this reaction was species dependent.

Hine *et al.* (1970) also reported that with exposures below 25 ppm the acute reactions were replaced by subtle changes located predominantly in the terminal bronchioles and alveoli. Freeman *et al.* (1969) described microscopic changes in the terminal bronchioles of rat lungs. Cilia were shortened or absent, and the normal replacement of bronchiolar epithelium was retarded. Chronic exposure increased interstitial connective tissue and decreased elastic recoil. Terminal bronchioles were narrowed, alveolar walls broken, and the lungs failed to collapse after removal from the thoracic cavity. The narrowing of the terminal bronchiolar lumen was due to epithelial hypertrophy, and accumulation of mucin, amorphous proteinaceous material, fibrin strands, and alveolar macrophages.

Freeman *et al.* (1969) reported that rats continuously exposed to 0.8 ppm nitrogen dioxide for up to 2 years had few lesions associated

with nitrogen dioxide toxicosis. The authors concluded that rats were unacceptable for long-term nitrogen dioxide toxicosis studies due to a short life span.

Purvis and Ehrlich (1964) described the effects of nitrogen dioxide inhalation on susceptibility to bacterial respiratory infections. Short-term, low-level exposure to nitrogen dioxide did not increase mortality, but levels greater than 5 ppm significantly increased mortality. Boren (1967) reported that nitrogen dioxide exposure impaired phagocytosis in the respiratory tract. Pearlman *et al.* (1971), in an epidemiologic survey of young children, reported higher incidences and more severe lower respiratory ailments in the more polluted areas of a metropolitan district.

Stokinger (1969), in an extensive review of the current environmental pollution status, reiterated the schema of Tappel, who proposed that the damage from oxidant gases was a sequela of lipoperoxidation of PUFA. Buell *et al.* (1965) proposed crosslinkage of collagen fibers and elastin as a result of lipoperoxidation. Thomas *et al.* (1968) reported increased pulmonary lipid peroxidation in rats following exposure to 1.0 ppm of nitrogen dioxide for 4 hours daily for 6 days. Pre-exposure treatment with massive doses of vitamin E (10 mg./rat/day) for 3 days partially reduced lipid peroxidation as measured by the production of conjugated dienes. Typical pulmonary changes associated with nitrogen dioxide exposure were found. Goldstein *et al.* (1970) reported decreased survival times in vitamin E deficient rats exposed to 10.4 ppm ozone compared to control rats on normal diets. The deficient rats died of pulmonary edema which the authors attributed to accelerated oxidation of PUFA, although no measurements for the oxidation products were reported. Repeated short-term exposures of 3.5 ppm ozone with

interspersed periods of convalescence produced decreased vitamin E values which the authors regarded as a sequela to the observed rise in pulmonary lipid peroxidation levels, although no measurements were reported. The effects of ozone, if any, on other tissues were not reported.

MATERIALS AND METHODS

General Experimental Plan

Forty-five weanling female, Sprague-Dawley (Spartan Research Laboratory)[a] rats, weighing 40 to 60 grams, were used in these experiments. Five animals were randomly assigned to each of the 9 treatment groups (Table 1).

Table 1. Characterization of treatment groups

Group No.	Housing	Diet	Vitamin E treatment (I.U./rat/wk.)	Nitrogen Dioxide treatment (ppm)
1	plastic cages	basal	0	0
2	plastic cages	basal	100	0
3	chamber	basal	0	0
4	chamber	basal	100	0
5	chamber	basal	0	30
6	chamber	basal	100	30
7	chamber	basal	0	20
8	chamber	basal	100	20
9	Necropsied on Day 1 of experiment			

[a]Haslett, Michigan.

Groups 1 and 2 were housed in conventional plastic cages with stainless steel tops. Groups 3 through 8 were maintained in individual cages constructed of 1.27 centimeter square wire mesh, 9.0 centimeters wide, 12.0 centimeters high, and 23.0 centimeters deep. These cages were enclosed in a plexiglass isolator 61.0 centimeters wide, 61.0 centimeters high, and 91.4 centimeters deep, equipped with a circular port (diameter - 51.0 centimeters) located on each of the other 3 sides to permit manipulation of the animals during exposures (Figure 3). A negative pressure system was assembled to draw the air through the chamber at a flow rate of 40.0 l. per minute. The point of entry of the gas was in the upper left corner and the exhaust outlet was located diagonally in the lower right corner as recommended by the U.S. Public Health Service Monograph, 57, (1959).

Nitrogen dioxide was obtained in a concentrated form (30,000 ppm NO_2 in air)[b] and was released from the pressurized cylinder at 30.0 p.s.i. into a Matheson 610 flowmeter[c] (Figure 4). Concentration of nitrogen dioxide in the chamber was monitored 3 times daily from the exhaust port by the Greissman-Saltzman technique (1968). Forty milliliters of chamber air was drawn through 10.0 ml. of absorbing reagent to form an azo color complex, which was quantitated using a Spectronic 20[d] Spectrophotometer.

Rats were fed a basal diet of the formula reported by Porta *et al.* (1968). Chemical analysis found 1.7 I.U. of vitamin E per kilogram

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[b]Cryogenic Gas Company, Detroit, Michigan.

[c]Matheson Gas Company, Joliet, Illinois.

[d]Bausch and Lomb, Rochester, New York.

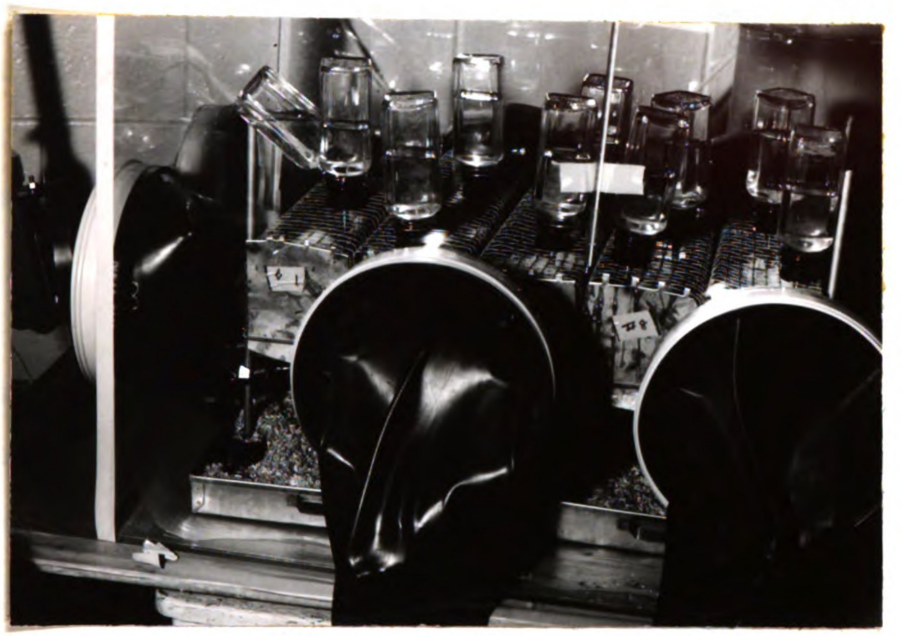

Figure 3. Individual rat cages within the plexiglass chamber used for this study.

Figure 4. Gas metering apparatus. Compressed gas cylinder (a), two-stage regulator (b), and capillary flow meter (c) which were used to meter NO_2 into the ambient air intake.

and 0.0243 ppm selenium in the formulated diet. The vitamin E supplemented groups received a weekly injection of 100.0 I.U. aqueous vitamin E/rat,[e] subcutaneously. Unsupplemented groups were given a 1.0 ml. injection of physiologic saline by the same route.

Groups 3 and 4 served as paired controls for Groups 7 and 8. Their feed consumption and time of death were governed by the action of their pairmates in Groups 7 and 8. Groups 5 through 8 were exposed to nitrogen dioxide continuously for 5 weeks. All rats which survived to termination dates were euthanatized by intraperitoneal injection of sodium pentobarbital. Necropsies were performed and the lungs and livers were weighed. The deflated lungs were gently perfused via the trachea with 10% formalin at 25 cm. of water pressure for 20 minutes and the trachea ligated. In addition to trachea and lung, portions of myocardium, liver, spleen, pancreas, kidney, adrenal, stomach, small intestine, and quadriceps muscle were collected in 10% buffered formalin for histopathologic examination. Other tissues were selected as gross examination warranted.

For routine histopathologic examination, tissues were stained with hematoxylin and eosin (H & E). Special stains, including periodic-acid-Schiff (PAS) stain for glycogen, Wilder's reticulum stain, Gomori's trichrome stain for collagen and smooth muscle, Verhoff's elastic stain, Mallory's phosphotungstic-acid hematoxylin stain for fibrin, and von Kossa's stain for calcium (Manual of Histologic and Special Staining Technics, Armed Forces Institute of Pathology, 1957) were applied as needed to clarify interpretations made by examination of the H & E

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[e]Hoffman LaRoche, Nutley, New Jersey.

preparations. Histopathologic examination of tissues was conducted in a semi-blind arrangement in which the author did not know whether the tissues were from vitamin E supplemented or unsupplemented animals.

In order to ascertain the pulmonary microbial flora of the rats used in this research, 5 rats were necropsied on Day 1. The bacterial culture technique consisted of aseptically removing a portion of lung tissue from each rat, finely mincing this tissue, and inoculating it into brain heart infusion (BHI) broth. The inoculated broth was maintained at 37 C. for 48 hours, at which time a loop of broth was transferred to a blood agar plate and streaked for individual colonies. The plates were observed at 24 and 48 hours for bacterial colonies. The lungs and ear canals were cultured for mycoplasmal infection in the following manner: A piece of lung was minced aseptically, as before, and placed in PPLO broth. One tenth milliliter of BHI broth was injected into the middle ear, withdrawn into a tuberculin syringe, and inoculated into PPLO broth. The cultures of the lung and ear canal were incubated for 72 to 96 hours, then inoculated onto PPLO agar plates and incubated for an additional 72 hours. At the end of 72 hours the culture plates were examined for colonies of *Mycoplasma sp.* Another tube of broth was inoculated from the original and the procedure was repeated 3 times for each sample.

Vitamin E Analysis

The livers of all rats were stored at -20 C. and were analyzed for tocopherol content. One gram of tissue was homogenized in a Potter-Elvehjem homogenizer with 10 ml. of absolute ethanol saturated with ascorbic acid. The homogenizer was washed with 2 10-ml. aliquots of absolute ethanol. The homogenate, the 2 washings, and 0.3 grams of

ascorbic acid were placed in a distillation flask for extraction and saponification. The ethanolic solution was heated for 5 minutes in boiling water to insure expulsion of oxygen from the flask, 1 ml. of 50% potassium hydroxide was added dropwise to the solution, and refluxing continued for 30 minutes. At the end of 30 minutes 0.9 ml. of 10% sulfuric acid was added to the reaction flask, the flask was removed from the heat source, immediately stoppered, and rapidly cooled in an ice water bath. The contents of the flask were poured into a separatory funnel containing 10 ml. of hexane, the distillation flask was washed with 10 ml. of 20% sulfuric acid and 5.0 ml. of hexane. The washings were added to the funnel, the funnel vigorously shaken, and the solvents allowed to separate. The water and the ethanolic fractions were removed. The hexane layer was washed with 10.0 ml. of 60% sulfuric acid, the contents shaken, and the aqueous layer removed. This procedure was repeated 3 times, and followed by a 10.0 ml. wash of 10% sulfuric acid. If the interface had not cleared completely with the last 10% acid wash, 1.0 ml. of absolute ethanol was added, the contents shaken, and the aqueous and ethanolic layers were removed. The remaining fraction was dried with sodium sulfate, filtered to remove the sodium sulfate, and dried under an atmosphere of carbon dioxide to complete dryness in a small flask. The residue was resuspended in hexane containing a known amount of hexadecyl palmitate, an internal standard, and maintained at -20 C. under carbon dioxide until determinations were performed on the gas-liquid chromatograph (GLC).

The recovery rate for this extraction procedure was examined by the addition of known amounts of alpha tocopherol standard to rat liver before homogenization. A recovery of 87.8% of the theoretical tocopherol content was obtained. The accuracy as determined by 5 repetitive

determinations of standard alpha tocopherol preparation was 99.3 ± 2.8%. The specificity of this procedure, as determined by comparison of retention times with a standard alpha tocopherol control, was excellent, although at the highest sensitivity ($1x10^{-12}$ amperes/millivolt) and attenuation factors, large amounts of background deflection were observed. This affected the specificity very little, but did hamper sensitivity to a degree. This problem became severe at the 100 µg./ml. level, but was corrected by increased 60% sulfuric acid washings during the extraction procedure.

The GLC procedure was that of Kovensky and Day (1971) with the following exceptions: The column temperature was 250 C., and 3 microliter injections were used. At tocopherol levels of 100 µg./ml., a sensitivity of $1x10^{-12}$ amperes per millivolt and a recorder attenuation factor of 4 was utilized. This sensitivity was adjusted as the concentration increased, and the deflections remained linear for a range of 100 µg./ml. to 100 mg./ml.

Statistical Analysis

A completely random design (CRD) with a 2x4 treatment factorial was the basis for the design of this experiment. Two levels of tocopherol supplementation and 4 nitrogen dioxide concentrations were the treatments. The parameters were obtained in the following manner: The ratio of final to initial weight in grams for each rat of Groups 1 through 8 was calculated. Hepatic and lung weights, as percentages of final body weight, were calculated. Survival time in days for each rat was recorded. Hepatic tocopherol values in micrograms per gram of tissue for each rat of Groups 1 through 8 were divided by the mean tocopherol value for Group 9. The resultant ratio was used in the

statistical analysis. Statistical analysis of the data was by analysis of the variance and Tukey's (hsd) procedures (Steel and Torrie, 1960). The level of significance was chosen as $P<.05$.

RESULTS

Clinical Changes

Rats of Groups 1 through 4 appeared normal throughout the experiment. Animals of Groups 5 and 6 showed evidence of nasal and ocular irritation which decreased after several hours of exposure and almost disappeared after 24 hours. A roughened, unkempt, yellow-stained hair coat, and a generalized depression of activity were observed after several hours' exposure. Hyperpnea, dyspnea, and oral breathing increased as the exposure period progressed. All rats of Groups 5 and 6 appeared similarly affected irrespective of treatment.

Groups 7 and 8 developed behavioral and respiratory patterns similar to Groups 5 and 6, but over a longer period of time. As the exposure period lengthened, increased dyspnea was observed with oral breathing usually, but not consistently, preceding death by several hours. Reaction to stimuli decreased, both in length and strength of response, as exposure time progressed. Terminally, only a slight twitch of the ears was observed following a sharp noise on the outside of the chamber.

The mean death time in days was significantly affected by nitrogen dioxide concentration but not by tocopherol treatment within the groups (Table 2). Groups 1 and 2 were similar to each other, but lived longer than other groups. Groups 7 and 8 lived significantly longer than Groups 5 and 6, but approximately 50% less than control Groups 1 and 2. Groups 5 and 6 lived significantly less than all other groups.

Table 2. Effect of tocopherol supplementation and NO_2 exposure on mean time to death in rats

Group No.	NO_2 Concentration (ppm)	Vitamin E Supplementation	Mean time to death (days)*	S.E.
1	-	-	35	0
2	-	x	35	0
3	-	-	19.8	4.44
4	-	x	18.2	2.33
5	30	-	1.84	.02
6	30	x	1.84	.02
7	20	-	19.8	4.44
8	20	x	18.2	2.33

Comparison of Treatment Means

Group No.	5	6	4	8	7	3	1	2
Mean**	1.84	1.84	18.2	18.2	19.8	19.8	35	35

*Groups 1 to 4 were euthanatized in fulfillment of experimental design.

**All groups joined by the same line were not significantly different ($P<.05$).

Groups 2, 4, 6 and 8 exhibited signs of irritation at the sites of vitamin E injection. The irritation was characterized by vigorous scratching of the injection site for several minutes followed by intermittent scratching for the next 24 hours. The control animals exhibited no signs of irritation following an injection of physiologic saline by the same route.

Weight changes, recorded as a ratio of final to initial weights, were examined for Groups 1 through 8 (Table 3). The rats of Group 1 were significantly heavier than those of the other groups. Group 2 animals were significantly heavier than the rats of Groups 3 through 8, but lighter than those of Group 1. All other groups were not significantly different.

Table 3. Effect of vitamin E supplementation and NO_2 exposure on mean weight gains, expressed as a ratio of final to initial weights

Group No.	NO_2 Concentration (ppm)	Vitamin E Supplementation	Mean Weight Gains (Final/Initial Wt.)*	S.E.
1	-	-	2.55	.19
2	-	x	1.72	.14
3	-	-	1.01	.16
4	-	x	.89	.08
5	30	-	.88	.05
6	30	x	.87	.03
7	20	-	.99	.04
8	20	x	.81	.08

Comparison of Treatment

Group No.	8	6	5	4	7	3	2	1
Mean**	.81	.87	.88	.89	.99	1.01	1.72	2.55

*Groups 1 to 4 were euthanatized in fulfillment of experimental design.

**All groups joined by the same line were not significantly different ($P<.05$).

Gross Lesions

All of the animals of Groups 2, 3, 4 and 9 were free of recognizable gross lesions. Group 1 animals were free of gross lesions in all organs except the liver where 1 animal had slight centrolobular necrosis while the remainder in the group had mild to moderate hepatic congestion.

Groups 5 and 6 had macroscopic lesions restricted to the lungs, liver and adrenal glands. The pulmonary changes were primarily petechial to ecchymotic hemorrhage, severe edema of the trachea and lungs, and a failure of the lungs to collapse when the thorax was opened (Figure 5). The severity of pulmonary lesions was surprisingly consistent throughout Groups 5 and 6. The lung weights for Groups 5 and 6, expressed as a percentage of body weight, were significantly different from those of control animals, but not from those of each other (Table 4). The adrenal and hepatic changes were characterized by congestion. Hepatic weight in Groups 5 and 6, expressed as a percentage of body weight, was not significantly different from that of the controls (Table 5). Adrenal weights were not determined.

Groups 7 and 8 were less uniform in the appearance of macroscopic lesions than Groups 5 and 6. The pulmonary lesions consisted of moderate petechial and ecchymotic hemorrhages, moderate edema, decreased recoil after opening the thoracic cavity and, in 1 animal, 2 large abscesses with accompanying consolidation. The edema and hemorrhage appeared to decrease in severity as survival time increased, although no clear pattern was evident. Lung weights for Group 7 were significantly greater than those of Groups 1, 2, 3, 4 and 8, but not different from those of Groups 5 and 6. Group 8 lung weights were significantly less than those of Group 7, but were not different from those of Group 4, its pair group. Hepatic and adrenal changes were not observed.

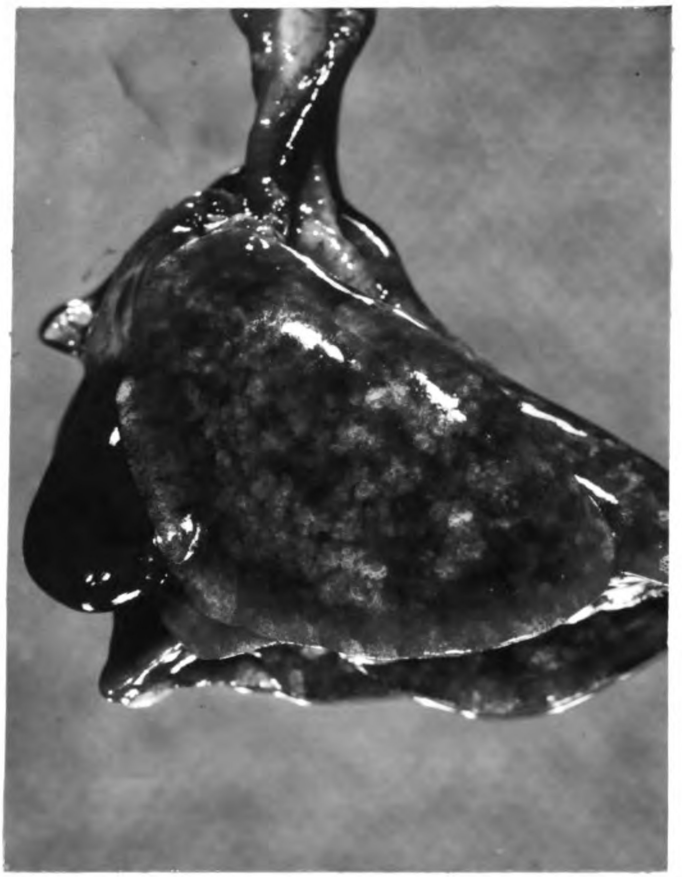

Figure 5. Lung with edema and petechial to ecchymotic hemorrhages illustrative of changes seen in rats exposed to 30 ppm NO_2 for 1.8 days (Group 5).

Table 4. Effect of vitamin E supplementation and NO_2 exposure on mean lung weights, expressed as a percentage of final body weight

Group No.	NO_2 Concentration (ppm)	Vitamin E Supplementation	Mean Lung Weight (Percent of final body weight)**	S.E.
1	-	-	.98	.13
2	-	x	1.16	.08
3	-	-	1.54	.25
4	-	x	1.66	.21
5	30	-	4.80	1.35
6	30	x	5.10	.83
7	20	-	5.44	.91
8	20	x	3.10	.20

Comparison of Treatment

Group No.	1	2	3	4	8	5	6	7
Mean*	.98	1.16	1.54	1.66	3.10	4.80	5.06	5.44

*All groups joined by the same line were not significantly different ($P<.05$).

**Groups 1 to 4 were euthanatized in fulfillment of experimental design.

Table 5. Effect of vitamin E supplementation and NO_2 exposure on mean hepatic weights, expressed as percentage of final body weight

Group No.	NO_2 Concentration (ppm)	Vitamin E Supplementation	Mean Hepatic Weight (Percent of final body weight)**	S.E.
1	-	-	5.00	.33
2	-	x	6.14	.53
3	-	-	6.12	1.17
4	-	x	6.58	1.10
5	30	-	6.00	.68
6	30	x	5.68	.79
7	20	-	5.78	.81
8	20	x	5.14	.57

Tukey's Procedure

Group No.	1	8	6	7	5	3	2	4
Mean*	5.00	5.14	5.68	5.78	6.00	6.12	6.14	6.58

*All groups joined by the same line were not significantly different ($P<.05$).

**Groups 1 to 4 were euthanatized in fulfillment of experimental design.

Hepatic weights were not significantly different from those of the other groups. Gastric ulceration was noted in 3 of 5 rats in Group 8. In all 3, free blood was observed in the stomach and upper small intestine but not in the remainder of the intestinal tract. All other tissues appeared normal.

Microscopic Changes

The tracheas and lungs from animals of Groups 1 and 2 were normal in appearance. The skeletal and cardiac musculatures, pancreases, intestines, stomachs, spleens and adrenal glands were normal for growing rats. Four rats, 2 from each group, had focal areas of calcification in the collecting tubules of the kidneys. The remainder of the renal structures appeared normal.

The livers of Group 2 animals were normal and contained large amounts of glycogen. The livers from 2 of 5 rats in Group 1 evidenced mild centrolobular necrosis characterized by pyknosis and loss of nuclei, decrease in staining quality, and disintegration of cell walls. No inflammatory response was present at the necrotic foci. The more severely affected rat had the most marked gross centrolobular changes. The livers of the other 3 appeared normal.

Groups 3 and 4 had no significant microscopic lesions in any of the organs examined.

In Groups 5 and 6 a number of lesions were observed. The tracheal changes included mild edema with a few mononuclear inflammatory cells in the submucosal areas, loss of cilia, and stunting and desquamation of mucosal cells. In the lungs, changes were similar in nature and severity to those seen in the trachea. Edema circling the bronchioles was variable in quantity and appearance with moderate numbers of mononuclear cells present in some areas (Figure 6). The predominant

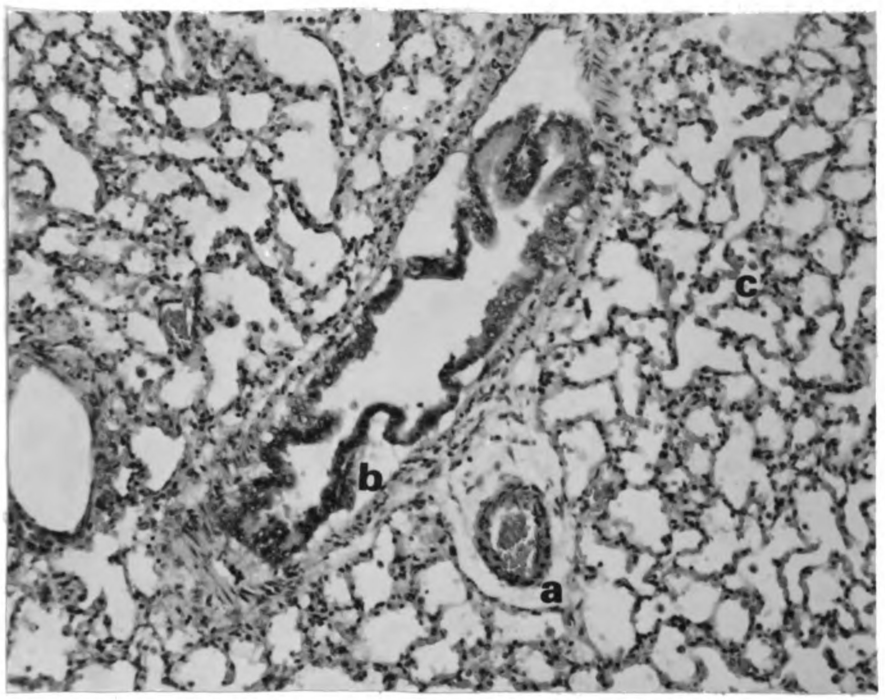

Figure 6. Perivascular edema (a), bronchiolar epithelial elevation due to edema (b), and capillary congestion (c) illustrating changes observed in rats exposed to 30 ppm NO_2 for 1.8 days (Groups 5 and 6). x60

pulmonary lesions were mild alveolar wall rupture and moderate alveolar thickening with increased cellularity primarily due to increased numbers of mononuclear cells. The alveoli were filled with a serofibrinous exudate which contained a small number of mononuclear cells. The vascular changes were striking, including large perivascular areas of edema (Figure 7), severe vascular congestion, and thickening and disruption of the endothelial lining of venules (Figure 8).

Skeletal and cardiac muscle changes included moderate to severe separation of fibers due to edema and slight hypercellularity (Figure 9). No other muscle lesions were noted.

Renal, adrenal and hepatic changes were characterized by severe vascular congestion and the absence of parenchymal damage. Splenic changes were moderate to severe congestion and increased extramedullary hematopoiesis as evidenced by increased numbers of erythrocytic precursors and megakaryocytes. The pancreas, stomach, and intestine were normal. The severity of lesions was not affected by treatment with tocopherols.

The microscopic lesions in rats of Groups 7 and 8 were variable in severity. Pulmonary lesions consisted of mild submucosal tracheal and bronchiolar edema with proliferation and folding of the bronchiolar epithelium (Figure 10), reduced number of ciliated epithelial cells and, in some cases, small to moderate amounts of cellular debris and mucous exudate in the bronchiolar lumina. Serofibrinous exudate and cellular infiltration were present around the bronchioles in large quantities. Thickening of the alveolar walls, primarily due to increased numbers of mononuclear inflammatory cells and neutrophils, rupture of alveolar septa, and accumulation of serofibrinous exudate were severe (Figure 11). In several areas this exudate was consolidated on

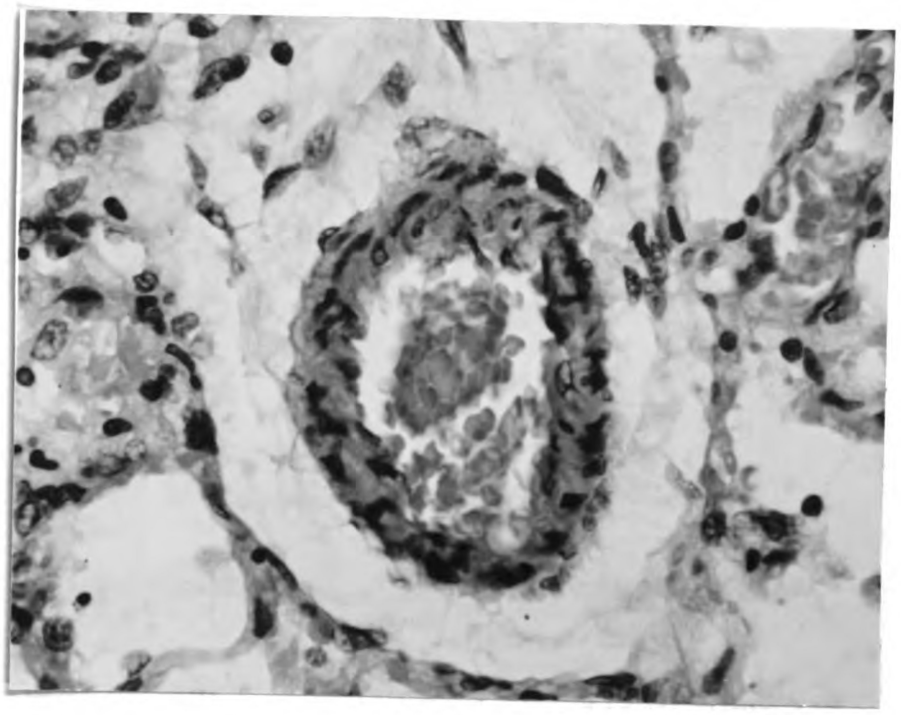

Figure 7. Pulmonary perivascular edema seen in rats exposed to 30 ppm NO_2 for 1.8 days (Group 5). x600

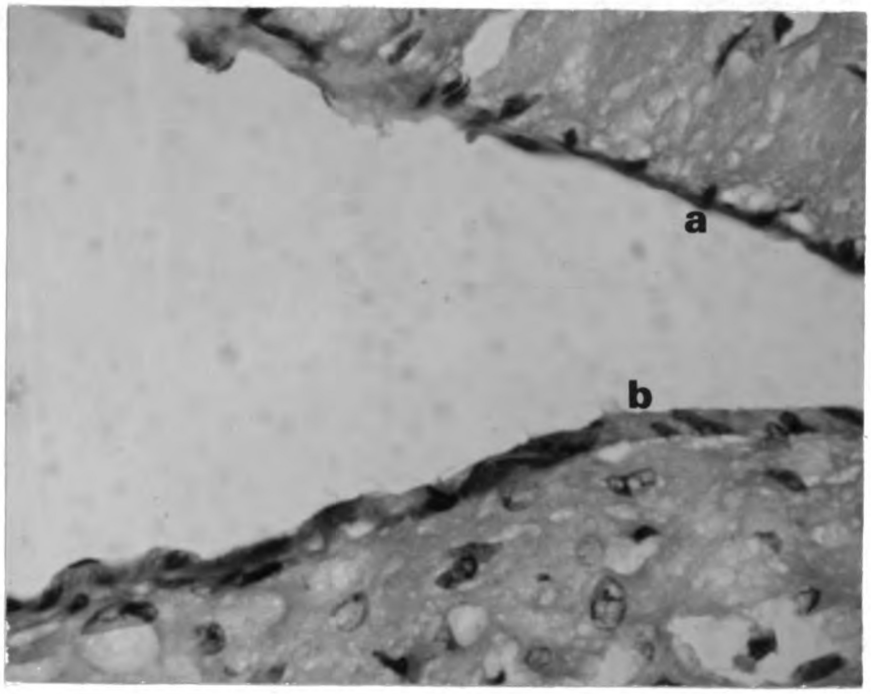

Figure 8. Pulmonary venule from a rat exposed to 30 ppm NO_2 for 1.8 days (Group 5). Normal endothelial lining (a) and thickened, elevated endothelial area (b) occurring in the same venule. x600

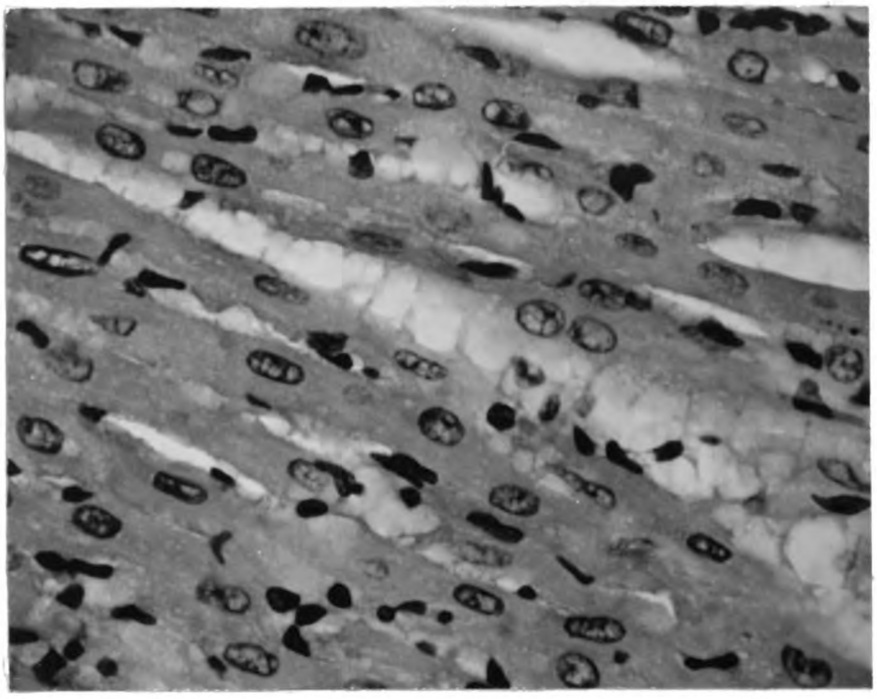

Figure 9. Typical myocardial lesions in a rat treated with 30 ppm NO_2 for 1.8 days. Separation of muscle fibers due to interfibrous edema. x600

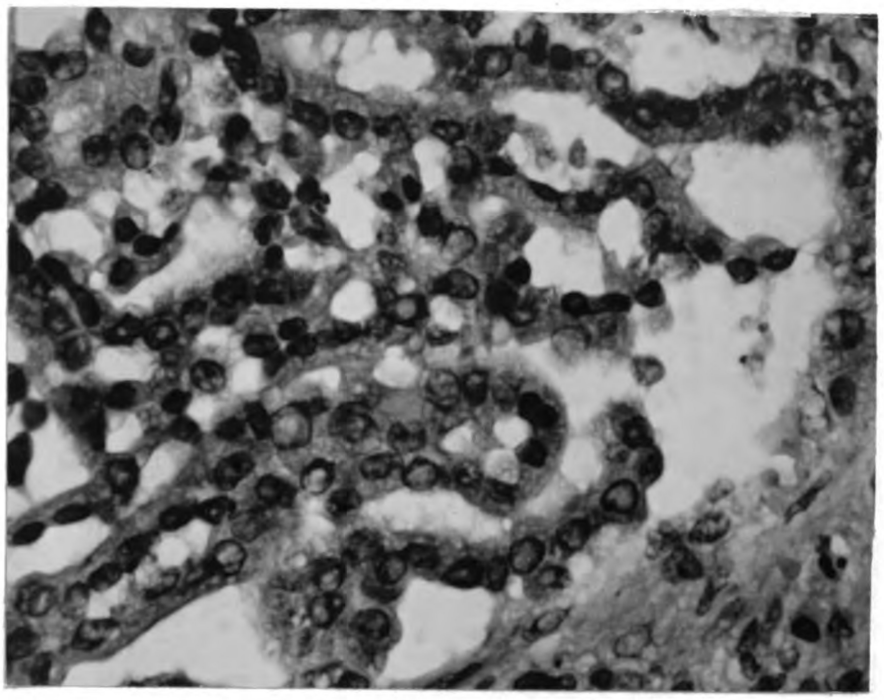

Figure 10. Bronchiolar proliferation in a rat exposed to 20 ppm NO_2 for 18.2 days (Group 7). x600

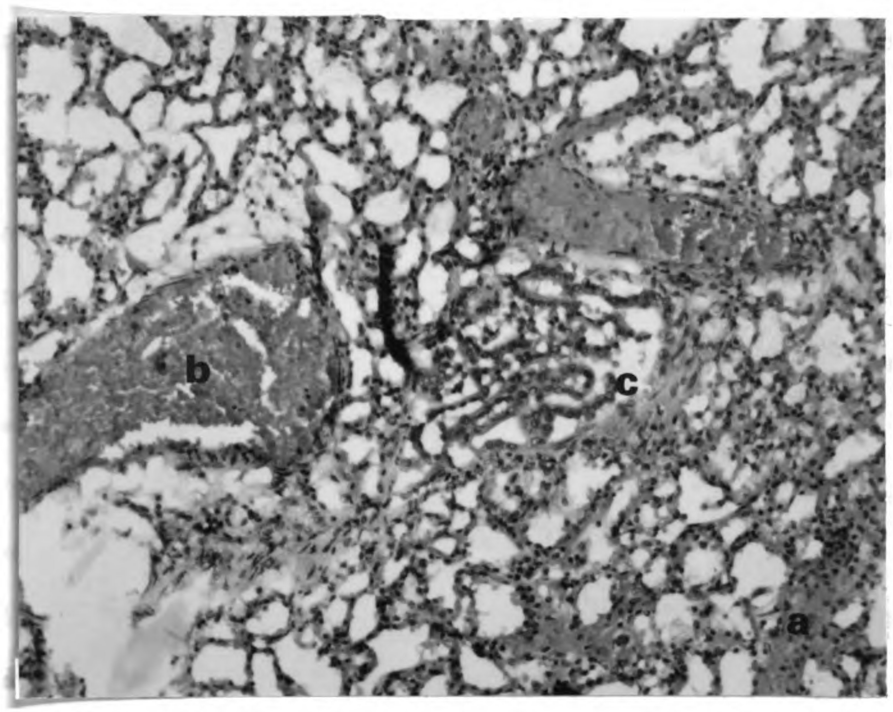

Figure 11. Pulmonary edema (a), vascular congestion (b), and bronchiolar proliferation (c) illustrating characteristic changes observed in rats exposed to 20 ppm NO_2 (Groups 7 and 8). x40

alveolar walls forming "hyaline-type" membranes. Alveolar hemorrhage was present but in very limited quantities. Vascular changes were milder than those of Groups 5 and 6 with smaller amounts of edema in the stroma of the vessels.

Two of 5 rats of Group 7 and 1 of 5 of Group 8 had microscopic evidence of suppurative pneumonia. The changes appeared to localize in 1 lobe while the other 4 remained unaffected. At the periphery of the larger areas of suppuration were small foci of inflammatory cells in the alveolar walls. These foci were variable in size and appeared to coalesce to form larger areas of suppuration. The inflammatory cells, primarily neutrophils, in these discrete foci appeared to be arranged in a radiating manner, and gave the impression of a "sunburst" appearance (Figure 12). This pattern was consistently present in the 3 infected animals, but was not observed in the controls or the other exposed animals in their groups. A Gram stain revealed abundant numbers of Gram-negative rods in all areas of inflammation, but not in other areas of the lungs.

Skeletal and cardiac musculature had mild separations of fibers by edema fluid. No signs of nutritional myopathy were observed in either tissue.

Mild congestion of parenchymal vessels was the only abnormality observed in the kidneys, adrenal glands, and liver. The splenic lesions consisted of moderate hyperemia, severe lymphoid depletion, and an absence of the increased extramedullary hematopoiesis observed in Groups 5 and 6. The only gastric lesion was seen in 2 of the 3 animals which grossly had gastric ulceration, and consisted of a mild, non-inflammatory exudate in the submucosal portion of the stomach, which elevated the mucosal structures from the stroma. The pancreas and intestine were

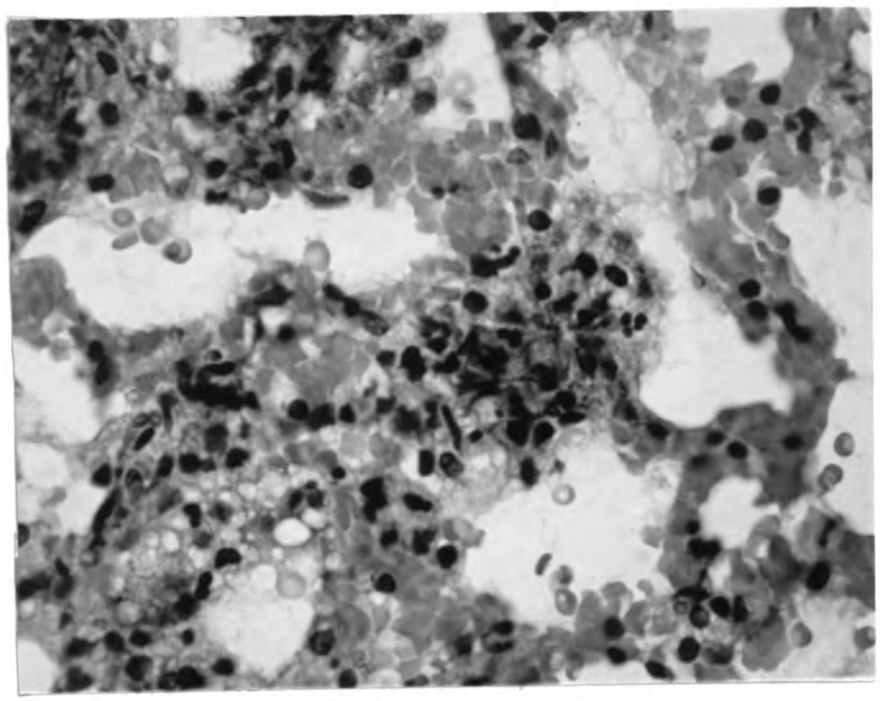

Figure 12. Focal bacterial pneumonia characteristic of the lesion seen in animals exposed to 20 ppm NO_2 (Group 7). x600

devoid of microscopic lesions. As with Groups 5 and 6, no distinction could be made between treatment Groups 7 and 8 with regard to the presence or severity of microscopic lesions.

Tocopherol Values

The hepatic tocopherol levels of the supplemented groups were significantly greater than those of the unsupplemented groups (Table 6). A significant difference between nitrogen dioxide treatment groups was not observed. The group means of 1, 3, 5 and 7 were not significantly different from each other but were significantly less than Groups 2, 4, 6 and 8. Groups 4, 6 and 8 were significantly greater than Group 2.

Bacterial Cultures

Cultures of the ear canal and lungs for *Mycoplasma sp.* in Group 9 were negative. Bacterial cultures of lungs from Group 9 were similarly nonproductive. At necropsy only 1 animal had gross indications of a bacterial pneumonia. Culture of the lung revealed *Pseudomonas sp.* in pure culture.

Table 6. Final mean hepatic tocopherol values, derived from the sum of ratios of individual hepatic tocopherol values within groups to a mean value of the controls killed on Day 1 (Group 9)

Group No.	NO_2 Concentration (ppm)	Vitamin E Supplementation	Liver Tocopherol Mean Values*	S.E.
1	-	-	.64	.13
2	-	x	10.23	4.20
3	-	-	1.07	.17
4	-	x	20.13	6.0
5	30	-	1.11	.14
6	30	x	26.53	5.89
7	20	-	1.08	.08
8	20	x	22.74	3.80

Comparison of Treatment

Group No.	1	3	7	5	2	4	8	6
Mean**	.64	1.07	1.08	1.11	10.23	20.13	22.74	26.53

*Groups 1 to 4 were euthanatized in fulfillment of experimental design.

**All groups joined by the same line were not significantly different ($P<.05$).

DISCUSSION

Clinical Changes

The nasal and ocular irritation observed in nitrogen dioxide exposed animals was consistent with the findings of others (Lillie, 1970). The signs of severe depression, respiratory distress, and anorexia in all nitrogen dioxide exposed animals were similar to those reported by Hine *et al.* (1970). The dose responsive effect, as measured by death time, was in accord with those of Steadman *et al.* (1966). The difference in survival times between 20 and 30 ppm appeared to substantiate the reports of Hine *et al.* (1970) that less than 25 ppm resulted in subacute to chronic changes while concentrations greater than 25 ppm caused acute death. The diet control animals, Group 1, survived until termination of the study at 5 weeks. This longevity was greater than that reported by Porta *et al.* (1968) on the same diet formulation. The presence of tocopherol and selenium in small amounts in the diet may have accounted for this variation. The weight differences among groups were related to their treatments. The animals of all groups, except 3 and 4, were fed *ad libitum*. Groups 3 and 4 were pair fed with Groups 7 and 8. The weight gains varied directly with consumption. Group 1 ate more than any other group. Group 2 animals ate less than those of Group 1 but more than any of the others. Groups 5, 6, 7 and 8 did not consume feed readily and consequently lost weight.

Gross Lesions

Gross lesions in Group 1 animals were milder than those reported by Schwarz (1965) as indicative of dietary hepatic necrosis. The absence of lesions in Group 2 was expected.

The lack of lesions in Groups 3 and 4 was as expected because of the short survival time in comparison to Group 1.

The pulmonary changes of Groups 5 and 6 were consistent with those reported by Hine *et al.* (1970). The vascular congestion and edema fluid in the parenchyma were responsible for increased lung weights over control animals. The hemorrhage resulted from vascular damage and was a consistent finding in this and other acute nitrogen dioxide exposures. The increased hepatic and adrenal congestion was probably a manifestation of generalized passive congestion resulting from increased capillary pressure in the lungs.

The reasons for variability in the severity of pulmonary lesions in Groups 7 and 8 was not clear, because all animals did not respond in similar fashion to the same gas concentration. Examination of the cage location failed to reveal a relationship between death time and area within the chamber. Gastric ulceration in 3 of 5 and pneumonia in 1 of 5 animals in Group 8 undoubtedly affected the death times of these animals to some extent. The appearance of pneumonia in 3 of 10 rats was consistent with the findings of Purvis and Ehrlich (1964). The reasons for localization in a single lobe and the formation of focal colonies which then spread within the lobe was not elucidated by this or other studies.

The lower lung weights for Group 8 rats when compared with Group 7 were an interesting observation. In all the nitrogen dioxide exposed groups the increased pulmonary weight resulted mainly from edematous

infiltration which was apparent microscopically. Although there was a significant difference in lung weights between rats of Groups 7 and 8, there was no difference in the degree of pulmonary edema apparent microscopically. One possible explanation for this decreased edematous infiltration could be an increased vascular integrity with tocopherol supplementation.

Microscopic Changes

The absence of microscopic changes in Groups 2, 3 and 4 was expected. The renal tubular calcification in 2 animals of Group 2 was considered insignificant. The hepatic lesions in 1 of 5 Group 1 animals indicated that the diet was deficient in vitamin E-selenium and that the development of hepatic necrosis was slower in this experiment than that of Porta *et al.* (1968).

The pulmonary changes of Groups 5 and 6 were consistent with those reported by Hine *et al.* (1970). The bronchiolitis, edema, vascular congestion and petechiation were undoubtedly due to the nitrogen dioxide exposure. Whether these results were due to simple irritation or membrane damage was not established. In this exposure group the nitrogen dioxide concentration was sufficiently large to mask small changes which might have served as clues to this problem. The endothelial thickening and disruption seen in this group could be illustrative of several very different mechanisms. The importance of this observation depends on the cause of the abnormality. If this endothelial disruption was caused by a lack of subendothelial support during tissue preparation due to the large quantities of edema separating the supporting structures, little significance can be placed on this observation. However, if this observation was not an artifactitious change, the thickening and disruption

of the endothelium would be significant. The edema and congestion observed in renal, adrenal, hepatic and muscular tissues were manifestations of generalized passive congestion. The increased splenic extramedullary hematopoiesis was an attempt to compensate for the lower oxygen diffusion from the alveoli.

The microscopic changes in the lungs of Groups 7 and 8 were similar to those reported by Freeman *et al.* (1969) and Haydon *et al.* (1965). A decrease in alveolar edema, apparently associated with length of survival, was observed. The loss of ciliated cells, the proliferation and folding of bronchiolar epithelial cells, the mucous exudate and the cellular debris were responsible for much of the alveolar wall rupture in the areas these pathways served. The increased number of epithelial cells, debris, and exudate acted to decrease the functional size of the bronchioles, while the decreased numbers of ciliated cells were unable to remove the accumulated debris as would occur in a healthy bronchiole.

The thickening of alveolar walls, increased cellular infiltration and accumulation of serofibrinous fluid were indications of cellular irritation and response to the irritant. The formation of "hyaline-type" membranes was possibly due to resorption of the edema fluid from the alveoli, leaving the fibrinous components unabsorbed. These fluids coalesced and formed a membranous coating on the intact walls. This membrane probably reduced oxygen diffusion and contributed to the anoxia which resulted in death of affected animals.

Vascular edema, skeletal and cardiac fibril separation, and congestion of kidneys, adrenal glands, and the liver, being milder in Groups 7 and 8, represented a milder and more chronic vascular insult than that observed in Groups 5 and 6. The splenic lymphoid depletion

was undoubtedly attributable to prolonged corticosteroid secretion due to the stress of nitrogen dioxide exposure. The absence of increased extramedullary hematopoiesis indicated the rats had regained homeostasis following the deficit observed during the acute exposure period of Groups 5 and 6.

Tocopherol Values

The higher levels of tocopherol in the livers of the rats in the supplemented groups were not unexpected. The fact that the tocopherol values of Groups 1, 3, 5 and 7 (all unsupplemented groups) were not significantly different indicated that hepatic tocopherol stores were affected by diet more than by nitrogen dioxide treatment. The Group 1 rats consumed considerably more diet than the other groups, and thereby helped to offset the tocopherol-selenium deficiencies of the diet. Although hepatic tocopherol levels of Group 1 were not significantly different from those of the others, they were approximately 2/3 those of the other unsupplemented groups. Had the diet been devoid of vitamin E and/or selenium, Group 1 hepatic levels could have been even lower. In the supplemented groups, all were significantly greater than Group 2. This probably is a reflection of the time of death following tocopherol injection. In Group 2, 7 days elapsed before the animals were euthanatized. In the other 3 groups, the animals died randomly throughout the 10-day period. Individual values, as expected, revealed this trend.

The fact that no distinction could be made between tocopherol deficient and supplemented rats, when exposed to nitrogen dioxide, may have resulted from several factors. The level of nitrogen dioxide could have been too high to allow a difference in response to manifest itself. The deaths of some animals from each treatment group from causes not

directly related to tocopherol treatment (pneumonia, gastric ulceration, etc.) made interpretation of treatment results difficult.

Conclusions

It was concluded that vitamin E supplementation had no beneficial effect on NO_2 toxicosis at the 20 and 30 ppm levels. At 20 ppm, rats unsupplemented with tocopherols had heavier lungs than their treated counterparts. This raised the possibility that at lower levels of NO_2 (i.e., 1, 5 or 10 ppm) a benefit could be realized with tocopherol supplementation. Further work, similar to this, will be required to fully investigate the significance of this observation.

SUMMARY

The effect of vitamin E supplementation on NO_2 exposure was determined. A torula yeast diet was fed to all test groups to hasten hepatic tocopherol depletion. Tocopherol supplementation was one 100 I.U./rat dose of alpha-tocopherol, subcutaneously, per week. Two NO_2 levels (20 and 30 ppm), administered 24 hours/day until all rats in their respective groups had died, were used to ascertain the possibility of a graded response.

Clinically, all rats not exposed to NO_2 were normal. Rats exposed to NO_2 exhibited signs of nasal, ocular, and respiratory irritation. The time of onset, severity of signs and death times were dose related. Tocopherol supplementation had no measurable effect on clinical signs or time of death.

Macroscopic lesions in rats not exposed to NO_2 but maintained on vitamin E deficient diets for 35 days were limited to hepatic congestion and, in 1 animal, hepatic necrosis. There were no gross lesions in non-exposed rats given 100 I.U. of vitamin E/rat/week, subcutaneously, regardless of time of euthanasia (i.e., at 18 or 35 days). Unsupplemented rats killed after only 18 days had no gross lesions. Gross changes in those exposed to NO_2 were pulmonary edema, congestion, and hemorrhage. These changes were more severe in the higher NO_2 exposure groups.

Microscopic changes in the unsupplemented diet control group were congestion of hepatic lobules and, in 1 animal, slight centrolobular hepatic necrosis. Sections from all other tissues appeared normal.

In rats exposed to 30 ppm NO_2 pulmonary changes consisted of perivascular, peribronchiolar, and alveolar edema, vascular congestion, loss of ciliated bronchiolar epithelium, and mild bronchiolitis. Bronchiolar proliferation, bronchiolitis, mild perivascular and peribronchiolar edema, emphysema, alveolar wall thickening, and pneumonia were of variable severity at 20 ppm NO_2.

Growth rate was markedly depressed by NO_2 exposure. Some depression of appetite was observed in tocopherol supplemented rats. Nitrogen dioxide exposure had no measurable effect on hepatic tocopherol levels in either the supplemented or unsupplemented rats. At 20 ppm, rats unsupplemented with tocopherol had heavier lungs than their treated counterparts.

It was concluded that tocopherol supplementation had no significant effect on NO_2 toxicosis at 20 and 30 ppm levels.

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