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THE PHARMACODYNAMICS OF CERTAIN ENDOGENOUS MAMMALIAN ANTIOXIDANTS DURING NO_2 EXPOSURE

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THE PHARMACODYNAMICS OF CERTAIN ENDOGENOUS MAMMALIAN ANTIOXIDANTS DURING NO_2 EXPOSURE

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FOREWORD

The benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

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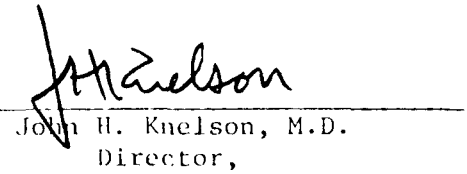
The assessment of the relative risk of an environmental hazard requires careful research and documentation of its adverse health related effects. This report documents the antioxidant role of vitamin E in preventing the peroxidation of polyunsaturated fatty acids by oxidizing atmospheres such as nitrogen dioxide and ozone. These fatty acids are constituents of cellular membranes, an alteration of which severely affects biological processes.



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ABSTRACT

Rats exposed to atmospheres containing nitrogen dioxide (NO_2) in excess of 10 ppm showed a 50% increase in uptake of ^{14}C-α-tocopherol by the lung when compared with control rats maintained in ambient air. This increase was not observed in liver or blood, the retention of ^{14}C-α-tocopherol being the same in exposed and control animals. NO_2 exposure did not affect the half-life of ^{14}C-α-tocopherol in lung, liver, or blood.

The liver of rats exposed to greater than 10 ppm NO_2 or to 1 ppm ozone showed a statistically significant ($P < 0.05$) increase in the level of α-tocopherol oxidation products compared with control rat liver, as judged by an increase in the ratio of α-tocopherol quinone plus α-tocopherol dimer to α-tocopherol. This increase was limited to the liver and was not observed in either lung or blood. Liver, lung, and blood of vitamin E-deficient rats exposed to 5 ppm NO_2 did not show any statistically significant increase in α-tocopherol oxidation products when compared with control tissues. No effect of NO_2 on ^{14}C-retinol acetate metabolism was observed.

This research resulted in the first description of an enzyme involved in α-tocopherol metabolism--namely, a UDP-glucuronic acid:dihydro-α-tocopheronolactone glucuronosyl transferase, the final enzyme in α-tocopherol metabolism before excretion. The glucuronosyl transferase is a microsomal enzyme found predominantly in the liver, and does not require a divalent cation for activity, although it is stimulated by Sn^{++}, Ca^{++}, and Mg^{++}.

This report was submitted in fulfillment of SRI Project LSU-3484, Contract No. 68-02-1713, by Stanford Research Institute under the sponsorship of the Environmental Protection Agency. Work was completed as of October 21, 1975.

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ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
BHT	Butylated hydroxytoluene
Ca^{++}	Calcium, divalent ion
^{14}C	Carbon 14
C	Celsuis
cm	Centimeter
Co^{++}	Cobalt, divalent ion
Cu^{++}	Copper, divalent ion
cu ft	Cubic feet
δ	Delta
L	Designates one of the two possible configurations about an asymmetric center
dpm	Disintegrations per minute
$FADH_2$	Flavin adenine dinucleotide, reduced form
γ	Gamma
g	Gram
g	Gravity
GlcUA	Glucuronic acid
>	Greater than
hr	Hour
in	Inch
ip	Intraperitoneal
kl	Kiloliter
<	Less than

Mn^{++}	Manganese, divalent ion
Mg^{++}	Magnesium, divalent ion
Km	Michaelis constant
μCi	Microcurie
μg	Microgram
μl	Microliter
μmole	Micromole
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
min	Minute
M	Molar
nmole	Nanomole
Ni^{++}	Nickel, divalent ion
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
N_2	Nitrogen
NO_2	Nitrogen dioxide
O_3	Ozone
ppm	Part(s) per million
%	Percent
PO_4	Phosphate, inorganic
p	Probability
R_f	Relative migration rate
sq. in.	Square inch
[S]	Substrate concentration

TLC	Thin-layer chromatography
Sn^{++}	Tin, divalent ion
UDP	Uridine diphosphate
V	Velocity, initial
v/v	Volume per volume
Zn^{++}	Zinc, divalent ion

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We express our appreciation to Dr. William T. Colwell, Dr. Joseph I. DeGraw, Mr. Vernon H. Brown, and Miss Caroline SooHoo for the preparation of the ^{14}C-α-tocopherol and ^{14}C-retinol acetate.

SECTION I

CONCLUSIONS

The lung tissue of rats exposed to greater than 10 ppm NO_2 showed an approximately 50% increase in uptake of ^{14}C-α-tocopherol when compared with lung tissue of rats maintained in ambient air. This increase was not observed for other tissues, including liver and blood. NO_2 exposure did not affect the half-life of ^{14}C-α-tocopherol in lung, liver, or blood.

The liver of rats exposed to 10 or more ppm NO_2 or to 1 ppm ozone showed a statistically significant ($p < 0.05$) increase in the level of α-tocopherol oxidation products as compared with control rat liver. This was determined by comparing the ratio of α-tocopherol oxidation products (α-tocopherol quinone plus α-tocopherol dimer) to α-tocopherol. Use of this technique minimizes differences due to variable recoveries of α-tocopherol. The increase in the α-tocopherol oxidation products:α-tocopherol ratio for liver was not observed in either lung or blood at any NO_2 concentration tested, nor was it observed in liver, lung, or blood of vitamin E-deficient rats exposed to 5 ppm NO_2. Apparently, NO_2 exposure (10 or more ppm) causes increased oxidation of α-tocopherol. The oxidation products are then rapidly cleared from the tissue of origin and transported to the liver for subsequent metabolism and excretion.

No effect of NO_2 exposure on ^{14}C-retinol acetate metabolism was observed. However, because of the known lability of retinol compounds to light and oxygen and the attendant problem of assay, we cannot state with certainty that NO_2 exposure has any effect on retinol acetate metabolism.

One of the enzymes involved in α-tocopherol metabolism--namely, UDP-glucuronic acid:dihydro-α-tocopheronolactone glucuronosyl transferase--was also investigated. This enzyme catalyzes the transfer of glucuronic acid from UDP-glucuronic acid to reduced α-tocopheronolactone, the final step in α-tocopherol metabolism before excretion. The enzyme is found predominantly in the liver, with detectable levels also occurring in the kidney. Subcellular distribution studies suggest it is found in the microsomes (100,000 × g pellet). The glucuronosyl transferase has a K_m of approximately 2.8 mM for α-tocopheronolactone and 8 mM for UDP-glucuronic acid. Because of the ease with which dihydro-α-tocopheronolactone is oxidized, incubations were performed using α-tocopheronolactone as substrate. We found that microsomes contain NADH:α-tocopheronolactone reductase, which produces dihydro-α-tocopheronolactone. We also found that the liver of monkeys undergoing long-term NO_2 exposure (9 years, 2 to 9 ppm NO_2) shows a decreased level of glucuronosyl transferase activity. This is contrary to what was expected: NO_2 should increase α-tocopherol oxidation products, and we would expect to see increased levels of enzymes involved in α-tocopherol metabolism. Additional work is required to further substantiate this observation.

SECTION II

RECOMMENDATIONS

1. Examine urine, bile, and feces for the presence of ^{14}C-labeled compounds derived from ^{14}C-α-tocopherol.
2. Examine in more detail the kinetics of absorption, retention, and release of ^{14}C-α-tocopherol in response to NO_2 exposures.
3. Investigate the nature and relative quantities of ^{14}C-α-tocopherol excretion products with particular emphasis on dose-response relationship with NO_2 exposures.
4. Examine kidney and liver for enzymes capable of catalyzing the formation of α-tocopheronolactone glucuronide.
5. Further characterize the UDP-GlcUA:dihydro-α-tocopheronolactone glucuronosyl transferase, particularly for substrate specificity.
6. Characterize the enzymatic steps involved in the metabolism of α-tocopherolquinone to α-tocopheronolactone.
7. Carry out further studies on ^{14}C-retinol metabolism under more stringent conditions of atmospheric control.

SECTION III

INTRODUCTION

Vitamin E, a fat-soluble, antisterility factor, has been the subject of increasing research since its discovery by Evans and Bishop[1,2] 50 years ago. Although the basis for its action as an antisterility factor remains unexplained, numerous additional biological functions have been ascribed to Vitamin E.

The predominant theory for vitamin E action is based on the antioxidant properties of the vitamin. Vitamin E prevents the peroxidation of polyunsaturated fatty acids found in lipids of cellular membranes, thereby stabilizing membrane structure. This action is demonstrated by the marked fragility of red blood cells of animals deficient in vitamin E.[3] Lipid peroxidation is initiated by exposure to hyperbaric oxygen and other oxidizing atmospheres such as nitrogen dioxide (NO_2) and ozone (O_3). Vitamin E is believed to quench highly toxic free radicals generated during peroxidation, thus terminating the free radical chain reaction.[4]

Several investigators have seriously questioned this role of vitamin E, principally regarding the quantitative relationships among α-tocopherols, the degree of peroxidation, and the appearance of lipid peroxides.[5] Recent research demonstrating the metabolism of lipid peroxides by glutathione peroxidase at the expense of NADPH counters the last objection.[6]

Other activities of vitamin E have been discovered. The vitamin has been demonstrated to affect the biosynthesis of heme by activating the initial controlling enzyme, δ-aminolevulinic acid synthetase.[7] In this role, vitamin E is involved in determining levels of hemoglobin and, hence, the oxygen-carrying capacity of red blood cells and levels of heme-containing cytochrome, thereby affecting mitochondrial electron transport and oxidative phosphorylation.[7] In addition, vitamin E has been reported to directly affect mitochondrial oxygen consumption[8] and to be involved in microsomal drug metabolism[9] and selenium metabolism.[5]

Very little is known about the enzymatic process governing α-tocopherol metabolism. One postulate is that, once α-tocopherol has been oxidized to α-tocopherolquinone, a sequence of enzymatic transformations occurs that results in the degradation of the isoprenyl side chain of α-tocopherol, yielding α-tocopheronolactone. Stumpf[10] has reviewed several postulated mechanisms, but enzymes capable of carrying out the required reactions have not been reported.

In 1912, Hopkins[11] identified vitamin A as a necessary nutrient required for normal growth. Subsequently, the plant pigment carotene was found to be an effective precursor to vitamin A that could be used as a dietary supplement in place of vitamin A.[12] Vitamin A is an extremely labile compound readily isomerized by light and oxidized by oxygen. This sensitivity to light is the basis for the biological role of vitamin A as a visual pigment in association with rhodopsin. Rosso et al.[13] demonstrated that

vitamin A also serves as a lipid-phosphate intermediate in certain glycosyl-transferase reactions.

Edwin et al.[14] demonstrated that vitamin E has a protective effect on vitamin A in biological tissues by preventing its oxidation. Therefore, because animals exposed to an oxidizing gas such as NO_2 exhibit decreased levels of vitamin E, a reasonable expectation is that secondary effects on vitamin A may also be observed.

This report describes studies on the effect of NO_2 on the retention of ^{14}C-α-tocopherol in lungs, liver, and blood and on the formation and disposition of α-tocopherol and retinol oxidation products. Also presented are initial results on the characterization of one of the enzymes involved in α-tocopherol metabolism, UDP-glucuronic acid:α-tocopheronolactone glucuronosyl transferase.

In these studies, rats were continuously exposed to subacute levels of NO_2 or O_3. Earlier studies[15-21] provided us knowledge of the well-defined sequence of morphological events arising from such exposure. During the first 24 hours, injury to and loss of both ciliated cells from the bronchiolar epithelium and Type I cells of the alveoli occur. Replacement of these cells by division of nonciliated and Type II cells begins and reaches a peak at the end of the second day. By the third day, the accumulation of cellular debris, fibrinous exudate, and macrophages and the hypertrophy of nonciliated cells cause obstruction of the small airways. After about 7 days of

continuous exposure, considerable repair has occurred, and the lung assumes a more normal appearance. However, further exposure causes additional cellular changes and invariably leads to the development of a disease resembling emphysema.

SECTION IV

MATERIALS AND METHODS

PREPARATION OF ^{14}C-α-TOCOPHEROL

To prepare α-tocopherol labeled with ^{14}C in the 5-methyl position, we used the method of Nakamura and Kijima.[22] Approximately 10 μCi (specific activity, 10 μCi/μmole) of ^{14}C-α-paraformaldehyde was reacted with 150 mg of γ-tocopherol. ^{14}C-α-tocopherol was isolated from the reaction mixture by thin-layer chromatography (TLC) on silica gel G plates in cyclohexane:ether (80:20, v/v). The radioactive band corresponding to α-tocopherol was eluted with ether, concentrated under N_2, and stored in redistilled benzene:ethanol (9:1, v/v) at -20°C until use. Before administration to rats, the ^{14}C-α-tocopherol was repurified by TLC as described above. This procedure resulted in ^{14}C-α-tocopherol preparations of at least 98% radiopurity.

PREPARATION OF ^{14}C-RETINOL ACETATE

To prepare ^{14}C-retinol acetate labeled in the 6-methyl group of the β-ionene ring, we used a combination of published procedures.[23-26] ^{14}C-Methyl iodide was reacted with 2,6-dimethyl cyclohexanone to yield 2,2,6-trimethyl cyclohexanone (2-methyl-^{14}C).[26] 2,2,6-Trimethyl cyclohexanone was then condensed with

3,7-dimethyl-4,6,8-nonatrien-1-yne-3-ol[23-25] to yield 3,7-dimethyl-1-(1-hydroxy-2,6,6-trimethyl-1-cyclohexyl)-3,5,7-nonatrien-1-yne-9-ol. Subsequent reduction, acetylation, and dehydration yielded vitamin A acetate.[24]

RATS

We obtained male Sprague-Dawley rats, age 30 days, from Hilltop Animal Farm, Scottdale, Pennsylvania. We chose this supplier because its rats were free of lung mycoplasma and other respiratory diseases. The rats were housed either in a control room, where they were exposed to ambient air, or in airflow chambers having an internal volume of 2.34 kl (82.5 cu ft). The rats in the airflow chambers were exposed to 5, 10, 14, or 20 ± 1 ppm NO_2 or to 1 ± 0.5 ppm O_3. The rate of airflow through the chambers was 1.13 kl/min (40 cu ft/min) at a slightly negative pressure of 0.19 to 0.36 mm of mercury (0.1 to 0.2 in. of water) below atmospheric pressure. Gas exposure chambers and generators have been described previously.[27,28] Rats were routinely fasted for 12 hours before being administered ^{14}C-α-tocopherol or ^{14}C-retinol acetate. Food was restored 4 hours postinjection.

ADMINISTRATION OF RADIOLABELED MATERIALS

^{14}C-α-Tocopherol

After exposure to NO_2 or O_3, groups of five rats each and five control rats were injected intraperitoneally (ip) with 1 to 3 μCi of ^{14}C-α-tocopherol in 0.5 ml of a 5% Tween 80 solution prepared under nitrogen with degassed water. In initial experiments, we injected 1 μCi of ^{3}H-L-Leucine with the ^{14}C-α-tocopherol

to follow the rate of protein synthesis. The exposed rats were returned to the NO_2 or O_3 chambers until sacrifice; control rats were maintained in ambient air.

^{14}C-Retinol Acetate

We used a procedure similar to that for administration of ^{14}C-α-tocopherol to give rats ip injections of ^{14}C-retinol acetate. The only difference was that the procedure was carried out in a photographic darkroom under red light. All solvents were deaerated before use. Storage and chromatography of retinol acetate were done under an argon atmosphere.

ASSAY FOR ^{14}C-α-TOCOPHEROL UPTAKE AND LEVEL OF OXIDATION PRODUCTS

All procedures were conducted in diminished light. Two methods were used to kill rats. The first was by ip injection of approximately 0.1 ml/100 g of body weight of a sodium pentobarbital solution (1 g/ml). This permitted the surgical exposure of the abdominal aorta and subsequent removal of a 3-ml blood sample with a heparinized syringe before organ removal. The second method was by decapitation and draining of blood into a heparinized centrifuge tube. The blood samples thus obtained were centrifuged for 10 minutes in a clinical centrifuge, and the plasma was decanted. Plasma obtained in this manner was used for subsequent analysis for ^{14}C-α-tocopherol and its oxidation products.

After blood collection, the liver and lungs were removed and immediately placed on ice. The assay method used depended on

whether we were investigating the total uptake and loss of α-tocopherol and metabolite or whether we were separating and quantitating α-tocopherol, oxidation products, and metabolites.

Total α-tocopherol and metabolites were estimated in the following manner: Approximately 3 g of tissue was homogenized in three volumes of normal saline (blood was diluted 1:4 with saline), and aliquots were assayed for protein. The aliquots were absorbed on a 1 sq. in. piece of filter paper, dried, and extracted with chloroform:methanol (2:1, v/v). The lipid extract was dried under N_2, and the ^{14}C-labeled content was quantitated by liquid scintillation spectrometry. The tissue content of α-tocopherol and α-tocopherol metabolites (^{14}C-labeled material) is expressed as disintegrations per minute (dpm) per milligram of protein or as total dpm per organ.

To determine the level of oxidation products of α-tocopherol (α-tocopherolquinone, α-tocopherol dimer and trimer), we used the following procedure. Approximately 1 g of liver or lung was finely minced and placed in the bottom of an 18 × 150 mm test tube; 2 ml of a 2% pyrogallol solution in 95% ethanol and 2 ml of saturated potassium hydroxide were added. Samples were hydrolyzed at 70°C for 30 minutes and cooled, 4 ml of water was added, and samples were extracted twice with 4 ml of 1.25 mM BHT in hexane. The combined hexane extracts were evaporated to dryness under a stream of nitrogen, dissolved in 0.2 ml of hexane, and subjected to TLC on silica gel G plates in cyclohexane: ether (80:20, v/v). The plates were allowed to develop to approximately 14 cm (in the dark). After evaporation of the

solvent, the plates were scraped in 1-cm bands and placed in scintillation vials; 5 ml of scintillation fluid was added, and radioactivity was determined in a liquid scintillation counter. After subtracting background, the total dpm corresponding to α-tocopherol, α-tocopherolquinone, α-tocopherol dimer, and α-tocopherol trimer was determined, and the ratio of α-tocopherol (dpm) to α-tocopherol oxidation products (dpm) was calculated.

ASSAY FOR ^{14}C-RETINOL ACETATE UPTAKE AND DISPOSITION IN TISSUES

Ten rats were exposed to 10 ppm of NO_2 for 3 days, injected under red light with 1.25 μCi of ^{14}C-retinol acetate in deaerated 5% Tween 80, and returned to chambers containing 10 ppm NO_2. Ten control rats were injected in the same manner and maintained in ambient air. Groups of five exposed and five control rats were killed at 24 and 72 hours postinjection. The liver, lungs, and blood were then removed in a darkened room. Approximately 1 g of each tissue was homogenized in one volume of distilled water, followed by homogenization with 5 ml of ethanol. Vitamin A was extracted from each tissue homogenate with cyclohexane containing 1.25 mM BHT and centrifuged to separate phases. The upper phase (cyclohexane) was removed and dried *in vacuo* in total darkness. The concentrated organic extract was rapidly spotted on silica gel G plates under red light in a darkroom and subjected to TLC in an argon-filled tank in cyclohexane: ether (80:20, v/v). After development, the thin-layer plates were dried and divided into 1-cm sections, each of which was

scraped into scintillation vials and counted for radioactivity by liquid scintillation spectrometry.

ASSAY OF UDP-GLUCURONIC ACID:DIHYDRO-α-TOCOPHERONOLACTONE GLUCURONOSYL TRANSFERASE

We used the method of Graham and Wood[29] to prepare rat liver microsomes. Freshly excised rat liver was homogenized in 10 volumes of cold 0.25 M sucrose for 40 seconds in a Waring blender. After centrifugation at 10,000 × g for 20 minutes, the supernatant was centrifuged at 80,000 × g for 90 minutes. The pellet was homogenized in 2 ml of 0.25 M sucrose per gram of fresh liver and centrifuged at 80,000 × g as above. The pellets were homogenized in 0.5 ml of 0.154 M of potassium chloride per gram of fresh liver and stored frozen in small aliquots. For the tissue distribution study, we obtained a crude enzyme preparation by homogenizing tissue in a Potter-Elvehjem apparatus with two volumes of 0.25 M sucrose.

Typical incubations contained the following materials in a total volume of 50 µl: α-tocopheronolactone (2.5 µmoles), NADH (5 µmoles), imidazole, pH 7.0 (5 µmoles), UDP-^{14}C-GlcUA (1 µmole), and microsomal enzyme protein (400 µg) or crude tissue homogenate (25 µl). After incubation at 37°C for 1 hour, 40 µl of the reaction mixture was spotted on Whatman 3-mm paper and chromatographed in ethanol:1 M ammonium acetate, pH 7.5 (7:3 v/v). α-Tocopheronolactone glucuronide migrated with an R_f of approximately 0.79 and was distinctly separated from UDP-GlcUA, GlcUA, and GlcUA-1-PO_4. The region containing dihydro-α-tocopheronolactone

glucuronide was cut out, and radioactivity was quantitated by liquid scintillation spectrometry.

SCINTILLATION FLUID

We prepared scintillation fluid by dissolving 22.74 g of PPO and 274 mg of POPOP per gallon of reagent-grade toluene.

PROTEIN

We used the method of Lowry et al.[30] to determine protein.

SECTION V

EXPERIMENTAL RESULTS

UPTAKE AND LOSS OF α-TOCOPHEROL

Figures 1, 2, and 3 show the retention of ^{14}C-α-tocopherol by the lungs, liver, and blood, respectively, of rats exposed to 14 ppm NO_2 for 3 days and of control rats exposed to ambient air. Both lungs and livers of NO_2-exposed animals retained more ^{14}C-α-tocopherol per gram of tissue protein than did those of controls. However, as indicated in Table 1, the lungs of NO_2-exposed animals were larger than those of control rats because of the incipient development of a disease resembling emphysema. Figure 4 presents the total ^{14}C-α-tocopherol content of the rat lungs. This determination shows that the lungs of NO_2-exposed rats, compared with those of control rats, retained a significantly greater amount of ^{14}C-α-tocopherol. In contrast, the livers of NO_2-exposed animals were smaller than those of controls (Table 1) so that, when the total ^{14}C-α-tocopherol content of liver was calculated, no difference between NO_2-exposed and control animals was observed as shown in Figure 5. The content of ^{14}C-α-tocopherol in the blood of exposed and control animals was the same at all times.

The rates of disappearance of ^{14}C-α-tocopherol from lungs, liver, and blood did not differ significantly between control and exposed rats (Figures 1, 2, and 3).

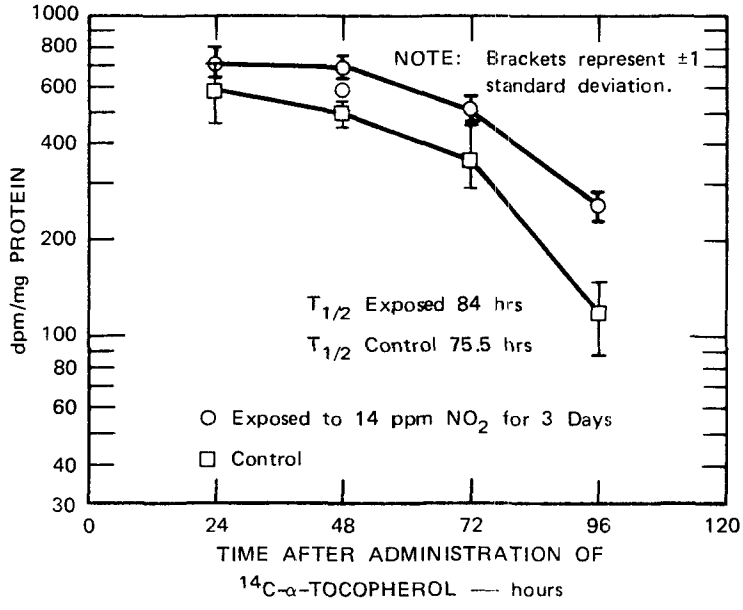


FIGURE 1 DISAPPEARANCE OF ^{14}C-α-TOCOPHEROL FROM RAT LUNG

Twenty rats were exposed to 14 ppm of NO_2 for 3 days and twenty rats were maintained in ambient air as controls as described in Materials and Methods. After 3 days, each rat received an ip injection of 3 μCi of ^{14}C-α-tocopherol (specificity, 10 μCi/μmole) suspended in aqueous 5% Tween 80. Exposed rats were returned to the chambers, and NO_2 exposure was continued. At the indicated time, five rats each from exposed and control groups were killed, and the total content of α-tocopherol and metabolites was determined.

As indicated in Table 2, comparison of the half-life of ^{14}C-α-tocopherol in NO_2-exposed and control lung, liver, and blood shows that the α-tocopherol is removed from the blood approximately twice as fast as it is from either the lungs or the liver. No significance is attached to the small difference observed in the half-life of ^{14}C-α-tocopherol in NO_2-exposed

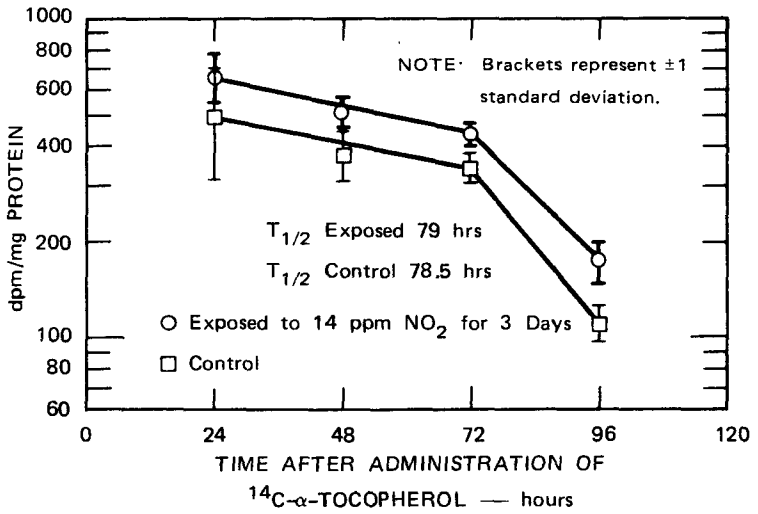


FIGURE 2 DISAPPEARANCE OF ^{14}C-α-TOCOPHEROL FROM RAT LIVER

Conditions were the same as those described in Figure 1.

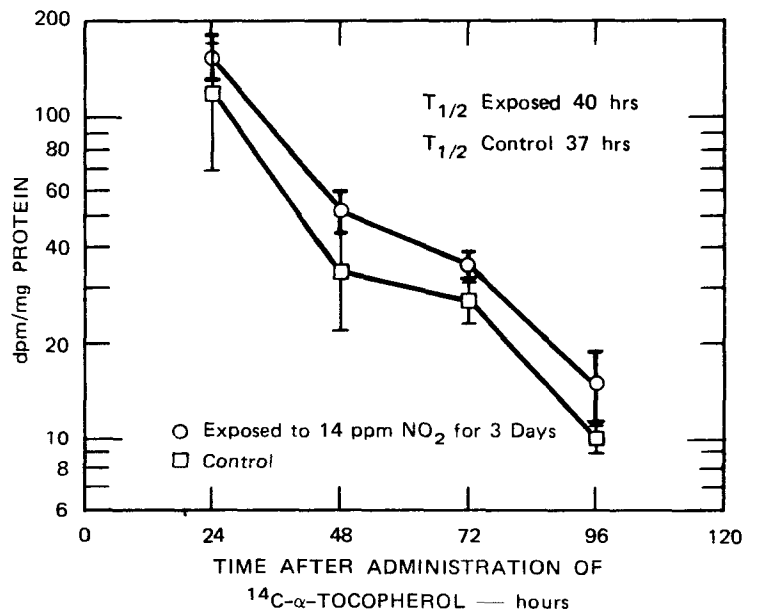


FIGURE 3 DISAPPEARANCE OF ^{14}C-α-TOCOPHEROL FROM RAT BLOOD

Conditions were the same as those described in Figure 1.

Table 1. TISSUE WEIGHT OF NO_2-EXPOSED AND CONTROL RATS[a] (grams)

Tissue	24 hr	48 hr	72 hr	96 hr
Lung				
NO_2 exposed	1.33 ± 0.19[b]	1.33 ± 0.15	1.48 ± 0.14	1.44 ± 0.04
Control	0.92 ± 0.08	1.06 ± 0.05	0.97 ± 0.05	1.11 ± 0.06
	$p < 0.005$[c]	$p < 0.01$	$p < 0.001$	$p < 0.001$
Liver				
NO_2 exposed	6.12 ± 0.90	5.51 ± 0.56	5.52 ± 0.47	5.47 ± 0.71
Control	8.57 ± 0.93	7.49 ± 0.50	7.35 ± 0.90	7.96 ± 0.27
	$p < 0.005$	$p < 0.001$	$p < 0.005$	$p < 0.005$

[a] Rats were exposed to 14 ppm of NO_2 for 3 days before administration of 3 μCi of ^{14}C-α-tocopherol in 5% Tween 80. Exposure was continued until sacrifice at the indicated times following ^{14}C-α-tocopherol administration.

[b] Tissue weight ± standard deviation.

[c] Standard Student's t-test.

and control lung tissue. Exposure to lower levels of NO_2 produced even smaller differences between exposed and control groups.

EFFECT OF NO_2 EXPOSURE ON TISSUE LEVELS OF α-TOCOPHEROL OXIDATION PRODUCTS

To determine whether NO_2 exposure affected tissue levels of α-tocopherol oxidation products, we injected rats previously

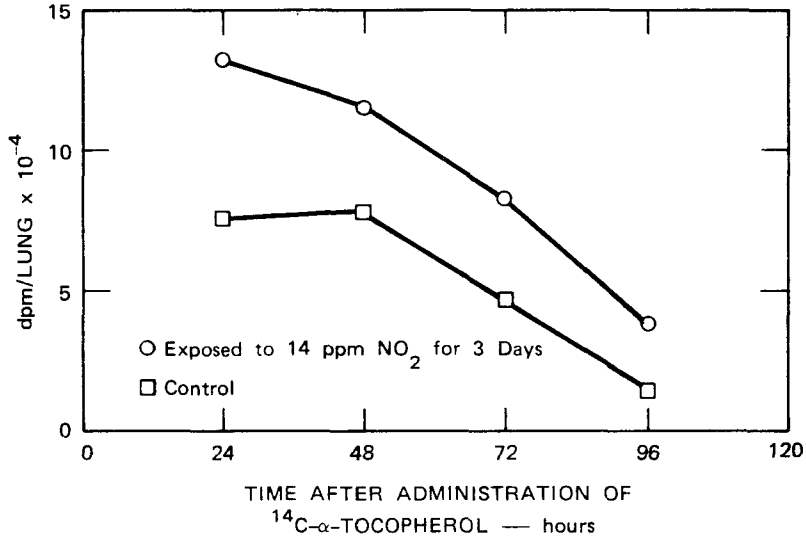


FIGURE 4 TOTAL ^{14}C-α-TOCOPHEROL CONTENT OF RAT LUNGS

Conditions were the same as those described in Figure 1.

exposed to 14 ppm NO_2 ip with 3 μCi of ^{14}C-α-tocopherol and continued the NO_2 exposure. At 24, 48, 72, and 96 hours after ^{14}C-α-tocopherol administration, we killed groups of five rats and removed lungs, liver, and blood as described in Materials and Methods. After TLC of the nonsaponifiable fraction, we determined the relative amounts of radioactivity corresponding to α-tocopherol, α-tocopherolquinone, and α-tocopherol dimer. Because α-tocopherol dimer and α-tocopherol trimer have similar R_f values in the solvent system used, and since little trimer was found as determined by mass spectrometry, only α-tocopherol dimer is reported.

is-C

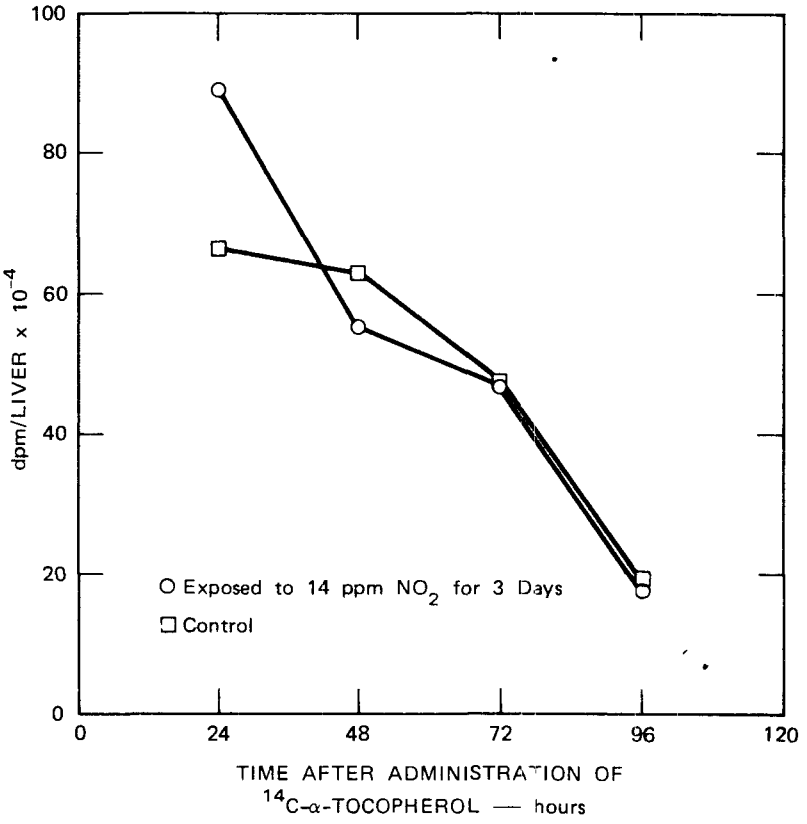


FIGURE 5 TOTAL ^{14}C-α-TOCOPHEROL CONTENT OF RAT LIVERS

Conditions were the same as those described in Figure 1.

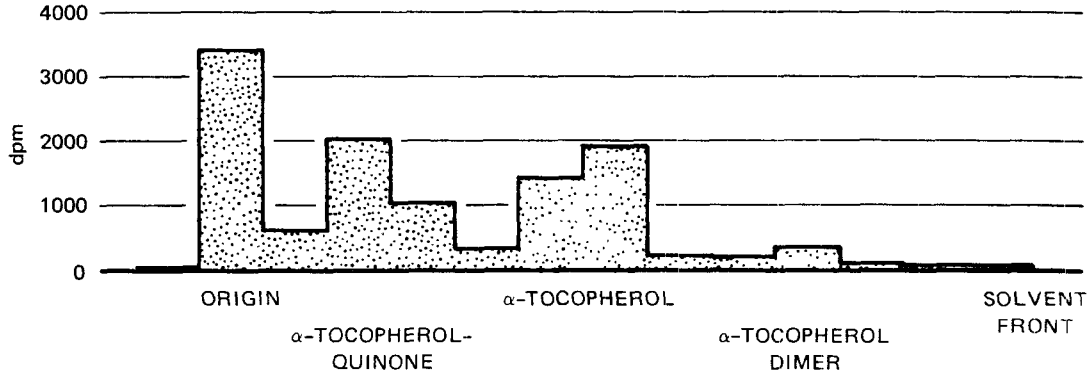
Figures 6 and 7 present typical results from lung and liver extracts from NO_2-exposed and control rats. α-Tocopherol, α-tocopherolquinone, and the dimer were readily separated from each other. A variable peak of radioactivity (up to 10% of the total) remained at the origin. This material is probably an oxidation product of α-tocopherol formed nonenzymatically during saponification; it was also formed when pure ^{14}C-α-tocopherol was subjected to base hydrolysis under the conditions used.

Table 2. HALF-LIFE OF ^{14}C-α-TOCOPHEROL IN TISSUES OF NO_2-EXPOSED AND CONTROL RATS[a] (hours)

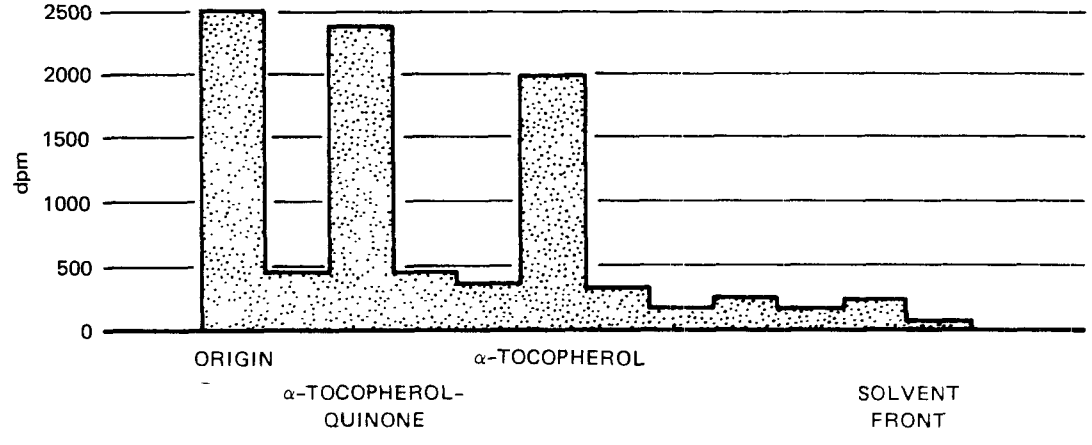
Tissue	NO_2 exposed	Control
Lung	84	76
Liver	79	78
Blood	40	37

[a]Half-lives for lung, liver, and blood were determined from data in Figures 1, 2, and 3. The length of time required for the level of ^{14}C-α-tocopherol to decline by 50% was determined from those data.

Its formation was minimized by including pyrogallol in the hydrolysis medium as an antioxidant. For these reasons, we included the radioactivity in the origin material with that of the α-tocopherol when we calculated the relative levels of tissue α-tocopherol. To circumvent the potential low recoveries in α-tocopherol assays, we determined the ratio of α-tocopherol oxidation products to α-tocopherol as a function of NO_2 exposure. When the data were expressed as the ratio of ^{14}C-α-tocopherol oxidation products (α-tocopherolquinone plus α-tocopherol dimer) to total ^{14}C-α-tocopherol (origin material plus α-tocopherol), no statistically significant difference in the ratio of α-tocopherol oxidation products to α-tocopherol was found in lung tissue.



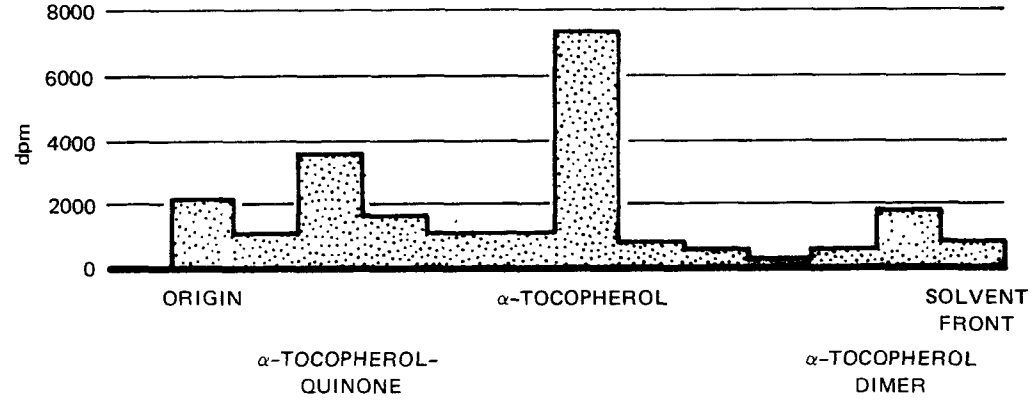
(a) LUNG EXPOSED TO 14 ppm NO_2 FOR 3 WEEKS



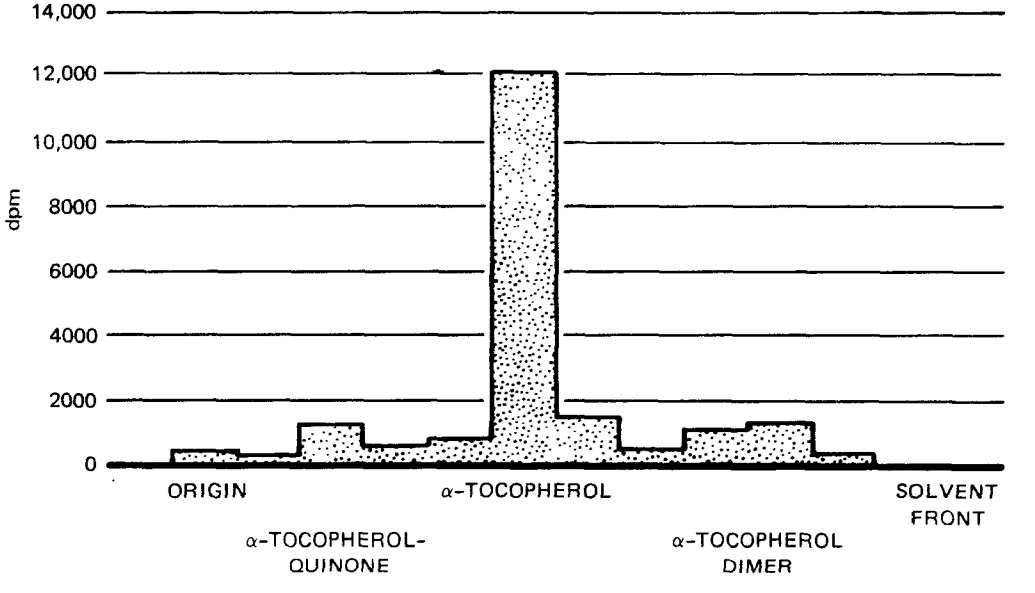
(b) LUNG CONTROL

FIGURE 6 THIN-LAYER CHROMATOGRAPHY OF ^{14}C-α-TOCOPHEROL AND METABOLITES FROM LUNGS OF NO_2-EXPOSED AND CONTROL RATS

Figure 8 shows these data. In contrast, a significantly higher ratio was observed in livers of rats exposed to NO_2 as compared with control rats, as shown in Figure 9. The proportion of oxidation products in the liver increased with time in both



(a) LIVER EXPOSED TO 14 ppm NO_2 FOR 3 WEEKS



(b) LIVER CONTROL

FIGURE 7 THIN-LAYER CHROMATOGRAPHY OF ^{14}C-α-TOCOPHEROL AND METABOLITES FROM LIVERS OF NO_2-EXPOSED AND CONTROL RATS

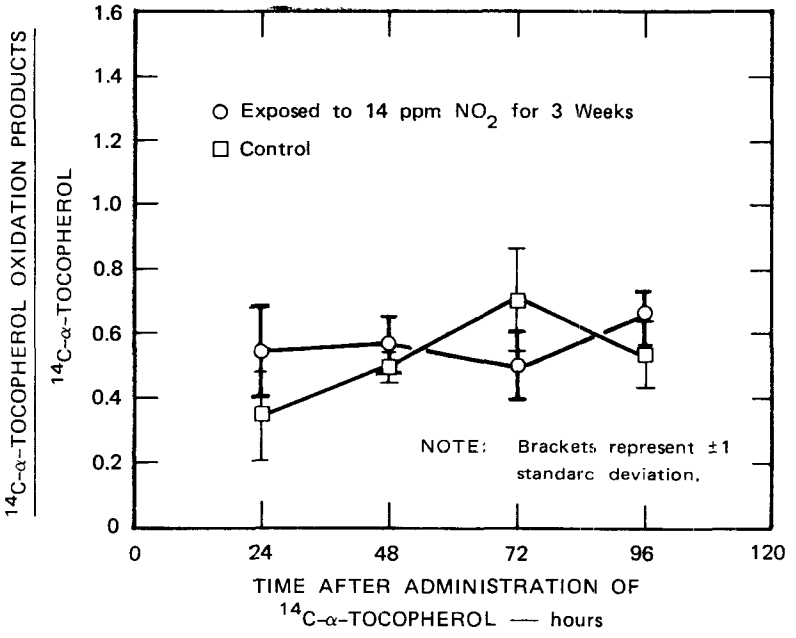


FIGURE 8 RATIO OF α-TOCOPHEROL IN EXPOSED AND CONTROL RAT LUNGS

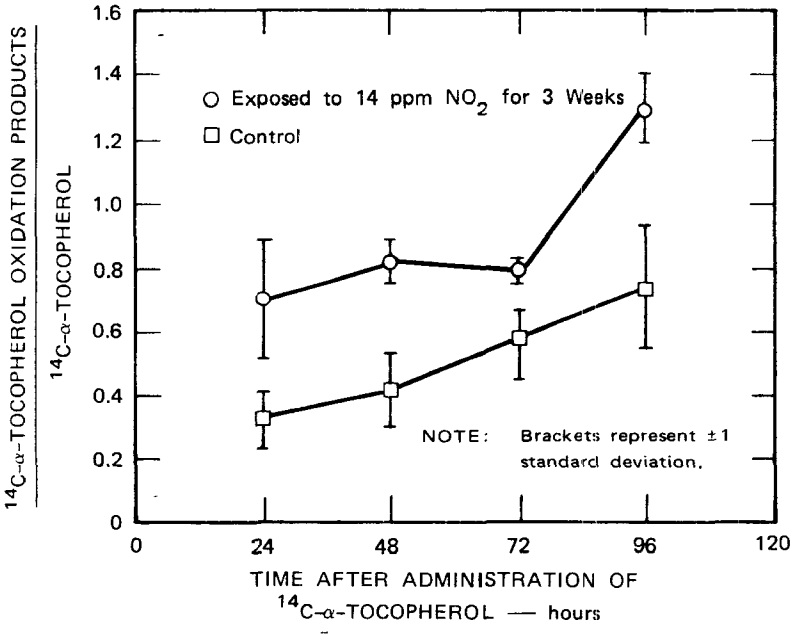


FIGURE 9 RATIO OF α-TOCOPHEROL OXIDATION PRODUCTS IN EXPOSED AND CONTROL RAT LIVER

exposed and control rats, although the rate of accumulation was somewhat greater in livers from NO_2-exposed rats.

As shown in Figure 10, we investigated the effects of different concentrations of NO_2 and of different exposure regimes on the ratio of α-tocopherol oxidation products to α-tocopherol in lungs, liver, and blood of the rat. Table 3 presents these data. One exposure experiment with O_3 was also performed. In all these exposure experiments, NO_2 and O_3 had no significant effect on the proportion of α-tocopherol oxidation products in the lungs. In blood, only the highest level of NO_2 used, 20 ppm, significantly increased the oxidation products.

In contrast, the livers had significantly elevated levels of oxidation products in all experiments except those in which the lowest level of NO_2, 5 ppm, was used. The proportion of oxidation products in livers from rats in the 10-day to 3.5-week exposure experiments was much higher than that in livers from animals exposed for 3 days. However, the levels of oxidation products in livers from animals exposed to 10 and 14 ppm NO_2 for about 3 weeks were higher than those in the corresponding controls. Only tissue from animals exposed to 5 ppm NO_2 had levels of oxidation products that did not differ significantly from the controls, even though these animals were deficient in vitamin E.

We conducted one experiment with exposure to 1 ppm O_2 for 3 days before ^{14}C-α-tocopherol administration. Table 3 shows that oxidation products were significantly high in the liver but not

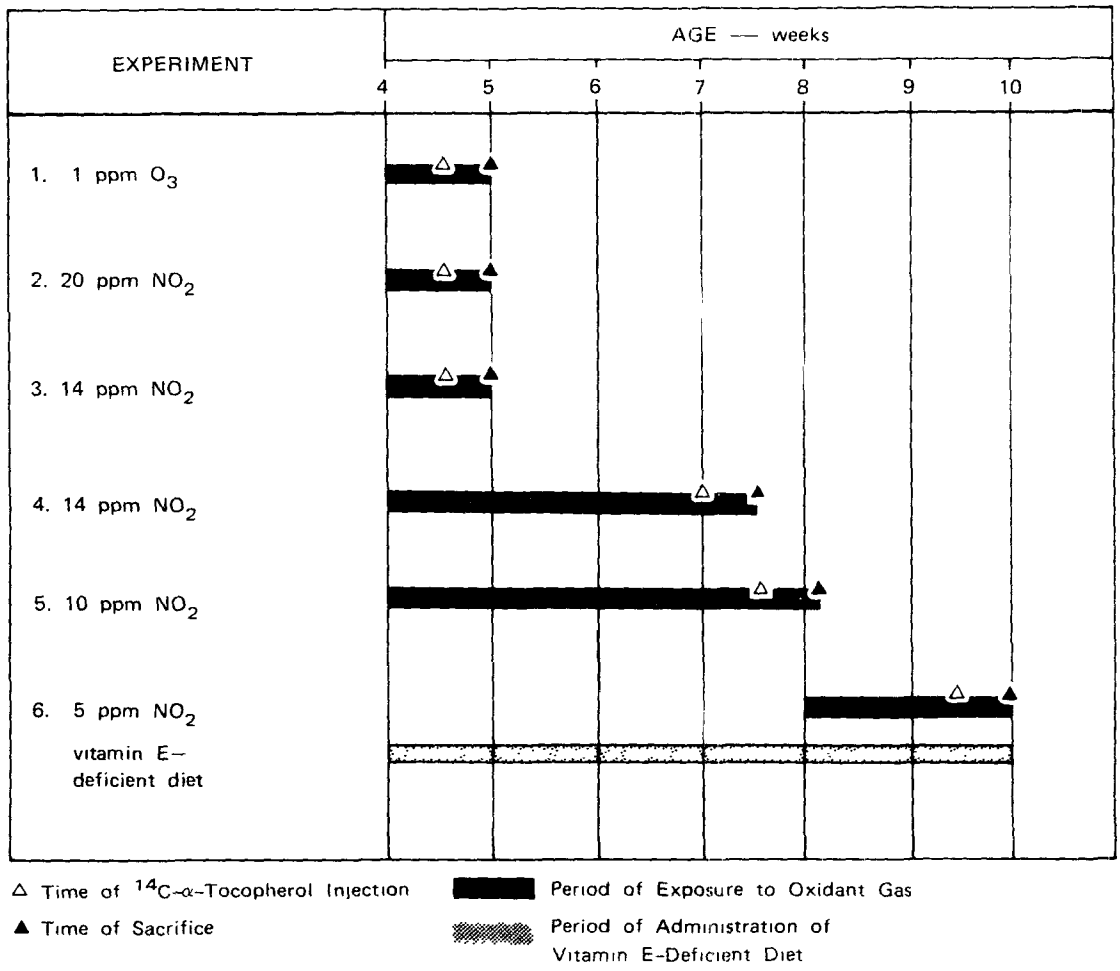


FIGURE 10 OXIDANT GAS EXPOSURE REGIMES

Experiments are identified and described in detail in the footnote to Table 3 (p. 27).

Table 3

RATIO OF α-TOCOPHEROL OXIDATION PRODUCTS TO α-TOCOPHEROL IN TISSUES OF OXIDANT GAS-EXPOSED AND CONTROL RATS[a]

Experiment No.	Initial Exposure	Animal Groups	Lung	Liver	Blood
1	1 ppm O_3 3 days	Exposed	0.40 ± 0.20	0.50 ± 0.15	0.39 ± 0.18
		Control	0.24 ± 0.12	0.26 ± 0.05	0.44 ± 0.23
			$p > 0.2$	$p < 0.05$[d]	$p > 0.5$
2	20 ppm NO_2 3 days	Exposed	0.81 ± 0.32	0.34 ± 0.15	0.34 ± 0.19
		Control	0.48 ± 0.28	0.15 ± 0.06	0.14 ± 0.04
			$p > 0.05$	$p < 0.05$	$p < 0.05$
3	14 ppm NO_2 3 days	Exposed	0.53 ± 0.18	0.31 ± 0.13	0.47 ± 0.22
		Control	0.18 ± 0.07	0.11 ± 0.03	0.34 ± 0.31
			$p < 0.025$	$p < 0.01$	$p > 0.04$
4	14 ppm NO_2 3 weeks	Exposed	0.66 ± 0.08	1.29 ± 0.12	--
		Control	0.54 ± 0.10	0.74 ± 0.22	--
			$p > 0.1$	$p < 0.005$	
5	10 ppm NO_2[b] 3-1/2 weeks	Exposed	0.63 ± 0.13	1.03 ± 0.17	--
		Control	0.65 ± 0.20	0.69 ± 0.15	--
			$p > 0.5$	$p < 0.05$	
6	5 ppm NO_2[c] 10 days	Exposed	0.63 ± 0.10	0.91 ± 0.39	1.29 ± 0.56
		Control	0.59 ± 0.12	1.24 ± 0.40	1.05 ± 0.67
			$p > 0.5$	$p > 0.4$	$p > 0.5$

[a]Weanling rats (age 30 days) were exposed to oxidant gas as indicated. ^{14}C-α-tocopherol was injected ip, and exposure was continued for 3 more days. Rats were killed, and the radioactivity in α-tocopherol and α-tocopherol oxidation products was determined as described in Materials and Methods. Tabulated values are the ratios of ^{14}α-tocopherol oxidation products to ^{14}C-α-tocopherol.

[b]Rats were killed 96 hours after administration of ^{14}C-α-tocopherol.

[c]Weanling rats (age 30 days) were placed on a vitamin E-deficient diet for 6 weeks. After 4 weeks on the diet, rats were exposed to 5 ppm of NO_2 for 10 days and then injected with ^{14}C-α-tocopherol. NO_2 exposure was continued, and 4 days later the rats were killed.

[d]Standard Student's t-test; values of $p < 0.05$ are considered to be statistically significant.

in lungs or blood. These results are similar to those obtained after 3 days of exposure to NO_2.

In some initial experiments, we injected 1 μCi of ^{3}H-L-leucine with the ^{14}C-α-tocopherol so as to monitor lung metabolism for use as a baseline reference. We found that the incorporation rate into protein was less than 100 dpm/mg, thus providing no advantage for ascertaining the state of the animal relative to metabolism of ^{14}C-α-tocopherol.

^{14}C-RETINOL ACETATE METABOLISM

We examined the metabolic fate of ^{14}C-retinol acetate by TLC of lipid extracts of tissues from NO_2-exposed and control rats. Figure 11 shows the results for liver. Only a single radioactive peak at R_f 0.78 was found. This material did not migrate with either retinol acetate (R_f 0.45) or retinol (R_f 0.10), and its identity remains unknown. Lungs, blood, and kidney from NO_2-exposed and control rats all showed similar results: only one peak of radioactivity at R_f 0.78 was observed. No significant differences between exposed and control animals were observed in the amounts of this material.

ISOLATION AND CHARACTERIZATION OF UDP-GLUCURONIC ACID:DIHYDRO-α-TOCOPHERONOLACTONE GLUCURONIC ACID TRANSFERASE

We assayed UDP-GlcUA:α-tocopheronolactone glucuronosyl transferase activity by following the transfer of ^{14}C-GlcUA from UDP-^{14}C-GlcUA to α-tocopheronolactone to yield dihydro-α-tocopheronolactone-^{14}C-glucuronide. The latter was readily isolated by chromatography

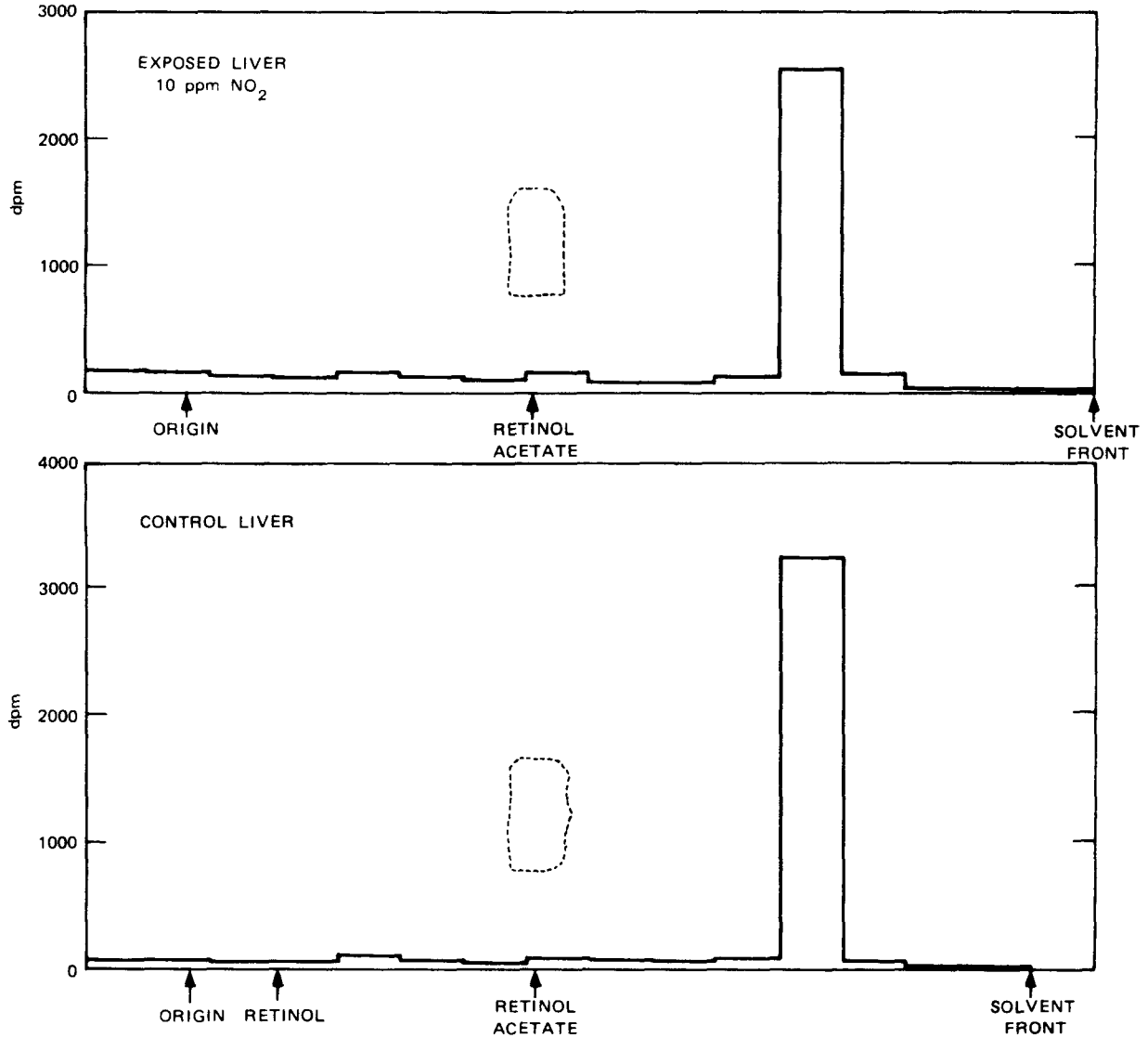


FIGURE 11 THIN-LAYER CHROMATOGRAPHY OF ^{14}C-RETINOL ACETATE AND METABOLITES FROM LAYERS OF NO_2-EXPOSED AND CONTROL RATS

Authentic retinol acetate is indicated by the hatched lines.

on Whatmann 3-mm paper using 95% ethanol:1 M ammonium acetate, pH 7.5 (7:3, v/v). Under these conditions, dihydro-α-tocopheronolactone-^{14}C-glucuronide migrated with an R_f of 0.79, whereas UDP-GlcUA, GlcUA-1-PO_4, and GlcUA migrated with an R_f of less than 0.5. Under the assay conditions described in Materials and Methods, incorporation of ^{14}C-GlcUA into dihydro-α-tocopheronolactone-^{14}C-glucuronide was linear with time for up to 1 hour and with enzyme protein for up to 0.7 mg per incubation (Figures 12 and 13). Glucuronosyltransferase activities are often linear for incubation periods of up to 6 hours or longer.[31] The initial rate of the reaction was somewhat low (Figure 12), presumably because of the requirement for reduction of α-tocopheronolactone to dihydro-α-tocopheronolactone before the addition of the glucuronic acid moiety. This was indicated by the finding that maximum

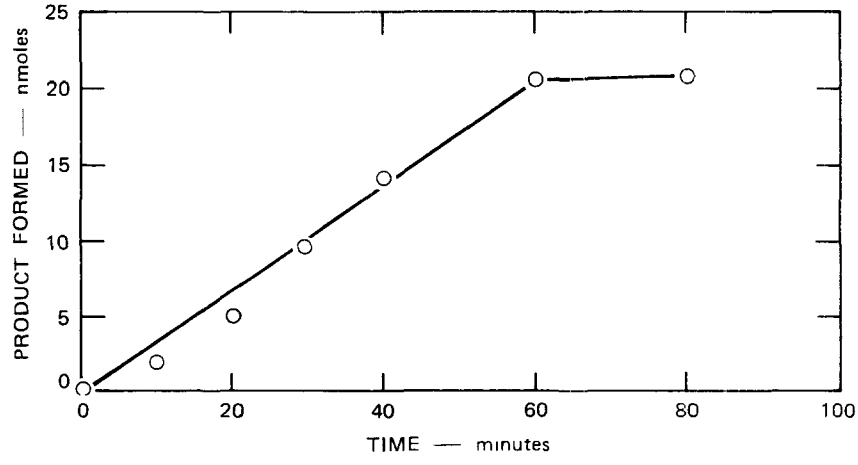


FIGURE 12 FORMATION OF α-TOCOPHERONOLACTONE GLUCURONIDE WITH TIME

Assays were performed using α-tocopheronolactone as substrate as described under Materials and Methods. Incubations contained approximately 0.5 mg of microsomal protein.

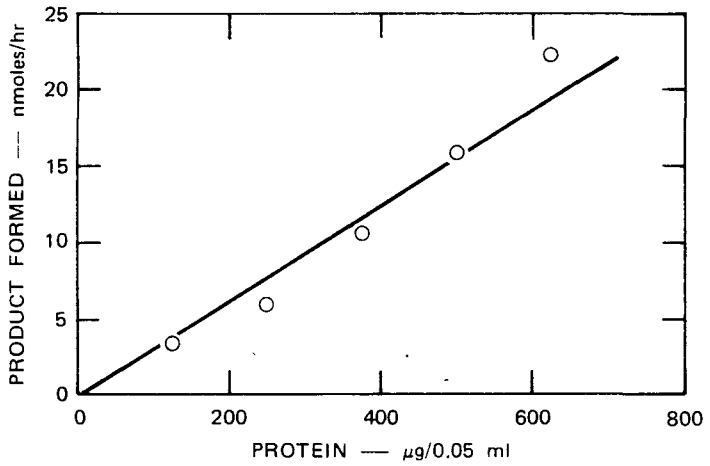


FIGURE 13 FORMATION OF α-TOCOPHERONOLACTONE GLUCURONIDE AT VARYING PROTEIN CONCENTRATIONS

incorporation occurred when NADH was included in the incubation as a potential cofactor for the reduction of α-tocopheronolactone. NADPH also stimulated the transferase reaction but at a lower rate, whereas $FADH_2$ had no effect.

As shown in Figure 14, the enzyme showed typical Michaelis-Menten kinetics using α-tocopheronolactone as substrate. A Km value for α-tocopheronolactone of approximately 2.8 mM was determined by the method of Lineweaver and Burke (Figure 14, inset). Substrate inhibition by α-tocopheronolactone was observed at concentrations of 50 mM or higher. We observed maximum incorporation of glucuronic acid into dihydro-α-tocopheronolactone glucuronide at a UDP-GlcUA concentration of approximately 0.01 M. The estimated Km value for UDP-GlcUA was 8 mM, as shown in Figure 15.

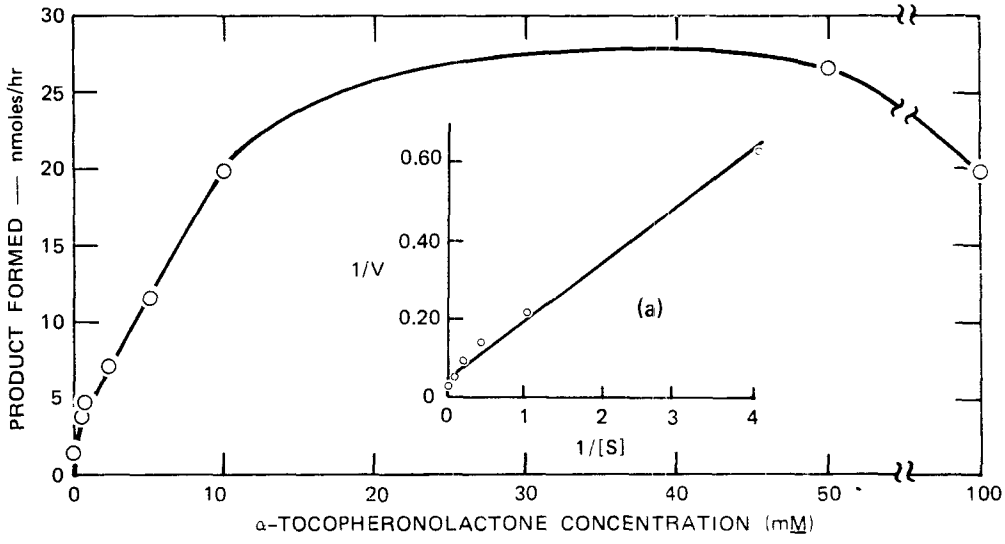


FIGURE 14 SATURATION OF GLUCURONOSYL TRANSFERASE WITH α-TOCOPHERONOLACTONE

(a) Determination of K_m for α-Tocopheronolactone by the Method of Lineweaver-Burke

The enzyme did not require divalent cations for catalytic activity. Most divalent cations tested (Co^{++}, Zn^{++}, Ni^{++}, Mn^{++}, Ca^{++}) inhibited enzyme activity, as indicated in Table 4, although Mn^{++} and Ca^{++} showed a slight stimulation at low concentrations (10 mM or less). Sn^{++} stimulated the enzyme reaction to the greatest extent (162% of control) but only at low concentrations (2 mM). Higher concentrations of Sn^{++} were not tested. These results suggest that divalent cations are not required for glucuronosyl transferase activity, although certain ones are stimulatory. Additional work is required to clarify this effect.

The glucuronosyl transferase activity is found predominantly in the liver of the rat, as shown by the tissue distribution study

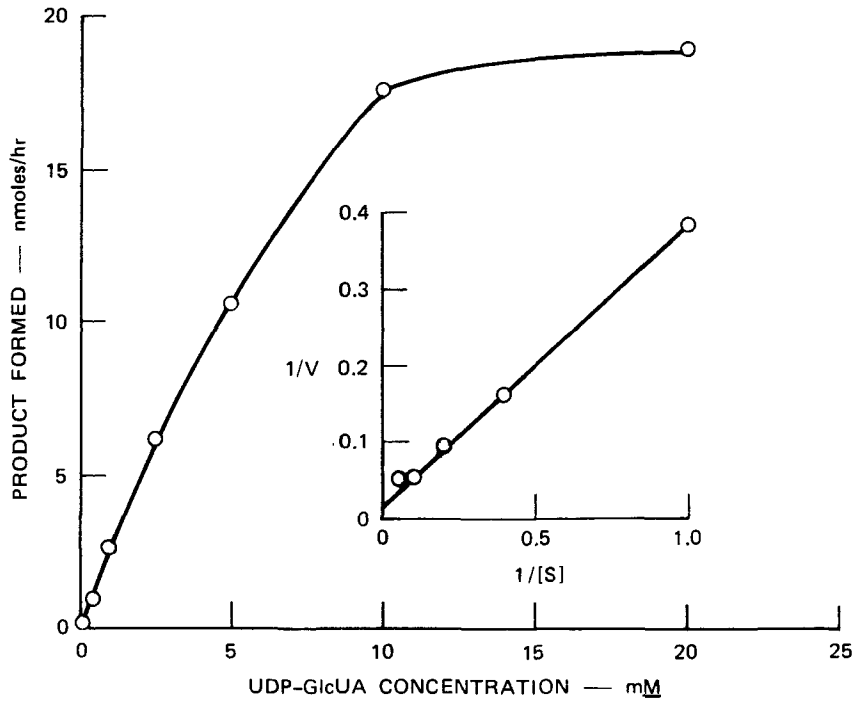


FIGURE 15 SATURATION OF GLUCURONOSYL TRANSFERASE WITH UDP-GlcUA

summarized in Table 5. Kidney has approximately 5% of the total activity of the liver, whereas spleen, brain, and lung have less than 1%. The activity of the enzyme in heart, fat, and serum was below the limits of detection.

During these studies, Dr. Gustave Freeman provided three samples of monkey (Macaca speciosa) liver from a control monkey and two monkeys exposed to 2 and 9 ppm NO_2 for approximately 9 years. We assayed these tissues for UDP-GlcUA:dihydro-α-tocopheronolactone glucuronosyl transferase, and Figure 16 presents the results. Enzyme activity was inversely proportional to the NO_2 concentration.

Table 4. EFFECT OF DIVALENT CATIONS ON GLUCURONOSYL TRANSFERASE ACTIVITY

Metal	Metal concentration, mM					
	2	5	10	25	50	100
Co^{++}		57	42		< 1	
Zn^{++}		73	38			
Sn^{++}	162					
Ni^{++}		59	18		0	
Mg^{++}		146	129		24	29
Ca^{++}		102	122		59	5
Mn^{++}		78	72	54	30	
Cu^{++}		0				

Metals were added as the dichloride salt to the standard transferase incubation mixture described in Materials and Methods. Tabulated values are percentage of transferase activity relative to control incubations lacking added metal.

Table 5. GLUCURONOSYL TRANSFERASE ACTIVITY IN RAT TISSUES

Tissue	Specific Activity[a]	Total Tissue Activity[b]
Liver	32.3	581
Kidney	7.7	26.8
Spleen	2.1	2.0
Brain	1.0	1.6
Lung	0.8	1.5
Heart	<0.2	<0.3
Fat	<0.2	--
Blood	0.8	--
Serum	<0.2	--

One unit of enzyme activity will convert 1 μmole of substrate per minute in the standard assay used.

[a] Units $\times 10^3$/g (ml) of fresh tissue.

[b] Units $\times 10^3$ per whole organ.

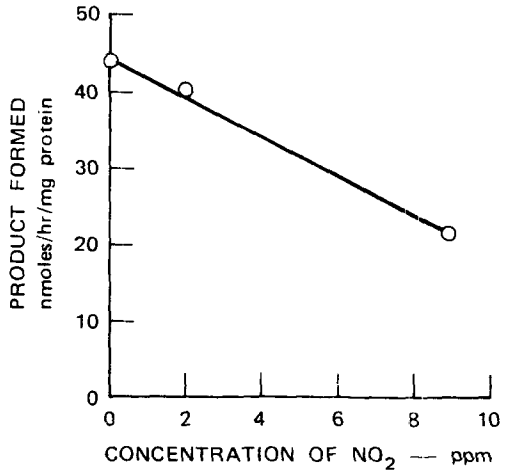


FIGURE 16 EFFECT OF NO_2 EXPOSURE ON GLUCURONOSYLTRANSFERASE ACTIVITY IN THREE SAMPLES OF MONKEY LIVER

SECTION VI

DISCUSSION

The objective of this project was to determine whether exposure of rats to an atmosphere containing NO_2 or O_3 has observable effects on the disposition and metabolism of vitamins E and A. Both these substances are susceptible to oxidation, and they may be oxidized in lung tissue *in situ* during exposure to NO_2 or O_3.

We have demonstrated that the uptake of ^{14}C-α-tocopherol in NO_2-exposed lungs is increased by approximately 50% over that in control lungs when measured either by uptake per milligram of lung protein (Figure 1) or by uptake in the total lung (Figure 4). This increase was not observed in liver and blood, the retention of α-tocopherol being the same for both exposed and control animals (Figures 2 and 5). NO_2 exposure did not affect the half-life of ^{14}C-α-tocopherol in lungs, liver, and blood. The ^{14}C-α-tocopherol was cleared from blood twice as fast as from lungs or liver (Table 2). These data suggest that lung tissue takes up an increased amount of the antioxidant α-tocopherol in response to exposure to an oxidizing atmosphere of NO_2.

We investigated the effects of different concentrations of NO_2 on the ratio of α-tocopherol oxidation products to α-tocopherol (Table 3). The livers of animals exposed to 10, 14, and 20 ppm

NO_2 had a significantly higher ratio of oxidation products than those from control animals. Blood had a significantly larger amount of oxidation products only in animals exposed to 20 ppm NO_2, whereas lung tissue did not show any significant difference in oxidation products for any exposure regime.

Because exposure to 14 and 20 ppm NO_2 had no effect on the amount of oxidation products found in lung tissue, and because we wanted to examine the effects of lower concentrations of NO_2, we used two stratagems in an attempt to accentuate the appearance of oxidation products. First we extended the initial exposure period to 3.5 weeks in the experiment using 10 ppm NO_2. As before, the lungs of the exposed rats had the same proportion of oxidized products as the controls. Also, the livers from the exposed rats had significantly larger amounts of oxidation products than the controls. This difference was similar to that found in the 14 ppm and 20 ppm exposure experiments.

The second technique was to deplete the endogenous stores of vitamin E in rat tissue by placing weanling rats on a vitamin E-deficient diet for 4.5 weeks before exposing them to 5 ppm NO_2, the lowest concentration of NO_2 used in these studies. Bieri[32] showed that weanling rats fed a vitamin E-deficient diet for 8 weeks still grew and displayed no overt signs of disease. At 4.5 and 8 weeks, respectively, the following percentages of α-tocopherol remained in various tissues: plasma, 1% and 1%; liver, 12% and 6%; fat, 28% and 10%; heart, 33% and 20%; skeletal muscle, 64% and 45%; and testis, 31% and 28%. Thus, from our experiment, which terminated at 6 weeks, we can surmise that,

when ^{14}C-α-tocopherol was injected after 5.5 weeks on diet, the stores of endogenous tocopherol in liver, fat, and plasma had been significantly depleted without pathological effects on the animals. Although the tocopherol content of heart was still dropping by 8 weeks, the levels in the testis and skeletal muscle had leveled off and were declining slowly. If the tocopherol content of lung tissue were influenced similarly to that of skeletal muscle or heart, we could assume that the content was below 50%. Under these circumstances, a reasonable expectation is that any vitamin E metabolism would engage a larger proportion of administered ^{14}C-α-tocopherol and, thus, appear amplified.

In contrast to results from previous experiments, the results of this experiment (Table 3) showed that the NO_2 exposure did not significantly alter the level of oxidation products compared with control values in any of the three tissues examined. Possibly, continuous exposure to 5 ppm NO_2 (and perhaps to even lower concentrations) for a longer time would result in the appearance of α-tocopherol oxidation products. However, in these experiments, the lowest level of NO_2 that enhanced the detection of α-tocopherol oxidation products was 10 ppm.

The results of exposure to 1 ppm O_3 were the same as those for exposure to 10 to 14 ppm NO_2. α-Tocopherol oxidation products were elevated in the liver, but the lung and blood were unaffected. These results are not unexpected, because O_3 at 1 ppm causes morphological and biochemical alterations of the lung that are very similar to those produced by NO_2 exposure at about 14 ppm.[33,34]

The chemical modes of action of the two gases are different,[35] and the effects they produce, although similar, can be readily distinguished by careful observation. For example, NO_2 exposure typically causes a doubling of glucose-6-phosphate dehydrogenase activity in lung,[36] whereas O_3 exposure increases the activity by only 50%.[6,36] Other biochemical differences can also be distinguished,[37] as can morphological differences.

During the first week of exposure, O_3 stimulates the infiltration of many more macrophages into the alveoli and of monocytes into the interstitial spaces than does NO_2. NO_2 tends to cause stratification of the nonciliated cells in the terminal bronchioles.[33,37] Long-term exposure to NO_2 (30 days or more) causes additional changes not seen with O_3. Proteinaceous crystalloids appear in nonciliated cells and later in ciliated cells.[33,38] Also, ciliated cytoplasmic vacuoles appear, basement lamina thicken, and the diameter of collagen fibrils increases.[39,40]

An unexpected result was that lung tissue of NO_2-exposed animals showed no significant differences in the levels of α-tocopherol oxidation products compared with lungs of control animals, whereas significant elevations were consistently observed in the liver (Table 3). This is particularly surprising considering that the exposed lung took up larger amounts of ^{14}C-α-tocopherol (Figure 4). We might expect that lung tissue--being directly exposed to the oxidizing gas NO_2 and having a greater retention of ^{14}C-α-tocopherol--would show the most pronounced effect on α-tocopherol metabolism. Instead, the products of α-tocopherol oxidation apparently are rapidly cleared from lung tissue and

are transported to the liver where they accumulate. This process continues for at least 4 days after administration of ^{14}C-α-tocopherol, since the level of α-tocopherol oxidation products present in the liver is still increasing at this time (Figure 9). The absence of a linear relationship between the concentration of NO_2 used in the exposure experiment and the level of α-tocopherol oxidation products found in the liver probably results from the relative rates of accumulation, subsequent metabolism including conjugation with glucuronic acid, and excretion. Other factors may also be involved such as artifactual oxidation of α-tocopherol or nonuniform response of animals to NO_2 exposure. Additional, more carefully controlled experiments are needed to delineate the system further.

Another interesting observation is that, in the three experiments involving long exposures to NO_2 (Table 3), the proportion of oxidation products found in the liver and blood of the control rats far exceeded that found in the control rats in the short-term experiments. The lungs, on the other hand, had the same proportion of oxidation products in all experiments. The only major difference between the two kinds of experiments is the age of the animals at the time of ^{14}C-α-tocopherol injection (Figure 10). In the short exposure experiments, the animals were about 4.5 weeks old, whereas in the long-term exposure experiments they were 7, 7.5, and 9.5 weeks old. A tempting speculation is that, as the animals age, larger amounts of α-tocopherol oxidation products are generated throughout the body and are accumulated in the liver for subsequent disposal.

The presence of α-tocopherol oxidation products in tissue has been noted before. In rats fed a vitamin E-deficient diet for several months, Peake and Bieri[41] found that 13% of ^{3}H-α-tocopherol appeared in the liver as oxidation products 27 hours after ip injection. Csallany et al.[42] recovered only 25% of total radioactivity in rat liver as unchanged α-tocopherol 2 days after injection of ^{14}C-α-tocopherol. In contrast, Krisnamurthy and Bieri[43] found that over 90% of orally administered tocopherol was recovered in rat liver regardless of the time interval up to 21 days after administration. Similarly, they found only 1% of the label from orally administered ^{14}C-α-tocopherol to be excreted in the urine, whereas Simon et al.[44] found that up to 30% of the label from ^{14}C-α-tocopherol administered intravenously to rabbits was excreted in the urine. Other urinary metabolites resulting from further side chain oxidation of α-tocopheronic acid have been described.[45] Clearly, the route of administration, the nutritional status of the animals, and the animal species used are important variables affecting the metabolism of α-tocopherol.

Good quantitation is difficult to obtain in vitamin E assays. Thus, researchers must take extreme precautions to prevent spurious oxidation of tocopherol during laboratory manipulations because of its sensitivity to oxidation by air and by tissue-derived heme compounds. In our work, routine precautions to minimize artifactual oxidation of α-tocopherol included working in a darkened room, using deaerated solvents containing antioxidants such as BHT or pyrogallol, and performing all

concentrations under an inert atmosphere of nitrogen. In spite of these precautions, the high proportion of ^{14}C-α-tocopherol recovered as oxidation products (up to 56% of recovered label, Table 3) may indicate artifactual oxidation. Nevertheless, we found (Table 3) that the ratios of oxidized products (quinone plus dimer) to unchanged α-tocopherol (α-tocopherol plus origin material) were reproducible within a single group of animals; but between groups, including both exposed and controls, considerable variation was observed. If this variation is artifactual it is curious that only in the groups of older animals were the ratios consistently higher than in the groups of younger animals. Oxidation products were formed in all tissue examined despite our efforts to minimize their formation by nonenzymatic artifactual means.

The enzymatic reactions in α-tocopherol metabolism have not been studied previously and none of the enzymes have been characterized. The appearance of increased levels of α-tocopherol oxidation products in liver due to oxidant gas exposure might induce an elevation in levels of enzymes involved in their subsequent metabolism. We chose UDP-GlcUA:α-dihydrotocopheronolactone glucuronosyl transferase for study because it is the last enyzme in α-tocopherol metabolism before excretion and because the glucuronic acid donor UDP-GlcUA and the potential acceptor α-tocopheronolactone were readily available. This enzyme catalyzes the transfer of glucuronic acid from UDP-GlcUA to reduced α-tocopheronolactone.

Our work demonstrates the presence of an active UDP-GlcUA:α-tocopheronolactone glucuronosyl transferase in liver, a slight activity in kidney, and negligible activity in the other tissues examined. This transferase does not require divalent cations as do some glucuronosyl transferases,[46] but it is slightly stimulated by Mg^{++}, Ca^{++}, and Sn^{++}. NADH also stimulates the activity, indicating a requirement for prior reduction of the lactone to the dihydrolactone because only the latter can be glucuronylated. Both activities are found in the microsomal fraction.

Liver contains UDP-glucuronic acid glucuronosyl transferase (acceptor-unspecific) (EC 2.4.1.17), which is responsible for the transfer of glucuronic acid from UDP-GlcUA to a variety of phenols, alcohols, amines, and fatty acids.[46] We do not know whether the glucuronosyl transferase activity using α-tocopheronolactone as substrate is due to the nonspecific glucuronosyl transferase because we did not perform substrate competition experiments.

Long-term exposure (9 years) of monkeys to NO_2 resulted in a decrease in the cellular content of transferase activity in the liver (Figure 16). This is surprising because higher levels of α-tocopherol oxidation products in the liver might be expected to produce an elevated enzyme level if any change were to occur. Because livers from only three monkeys were analyzed we cannot discount the possibility that the lower activities in the two exposed livers are due to biological variation.

In animal liver, lipid-free radicals or peroxides irreversibly oxidize α-tocopherol in small amounts to α-tocopherolquinone and

to dimeric and trimeric metabolites. The quinone itself is partially excreted in the feces, but most is reduced in the liver to the hydroquinone and conjugated with glucuronic acid and other unknown moieties before secretion in the bile and excretion in the feces.[47,48]

Trace amounts of α-tocopherol are converted to a conjugate of α-tocopheronic acid and excreted in the urine.[44] This material is presumed to arise by reduction of the quinone and conjugation with glucuronic acid and other substances in kidney.[48] Our finding that the bulk of UDP-GlcUA:α-tocopheronolactone glucuronosyl transferase activity resides in liver, with less than 5% activity in kidney, suggests instead that conjugation--at least with glucuronic acid--occurs in the liver. If this is the case, the degradation of the side chain most likely also occurs in the liver and the kidney probably serves primarily as the mechanism for the excretion of water-soluble metabolites.

However, we must consider that the glucuronide conjugate of α-tocopheronic acid comprised only about 5% of the urinary metabolites.[47] Another 45% of the metabolites were also acid hydroyzable, but these were shown not to be phosphate or sulfate esters.[47] Therefore, the possibility remains that the bulk of the α-tocopheronic acid derivatives excreted in the urine (about 90%) may be formed and conjugated in the kidney.

In this research, we demonstrated that oxidant gas exposure results in an increased rate of α-tocopherol oxidation. However, the tocopherol oxidation products, principally quinone and dimer,

are elevated only in the liver and at exposures to 10 ppm or higher of NO_2 or to 1 ppm O_3. Only at 20 ppm NO_2 did we observe enhanced levels of oxidation products in the blood. Oxidation products in the lungs were not elevated by any of the exposures. The amount of α-tocopherol in the lungs of exposed animals increased, whereas it remained the same in control animals.

These data suggest that lung damage caused by oxidant gas exposure leads to an increased turnover of α-tocopherol in the lung. Newly formed cells and cells undergoing repair require the importation of new α-tocopherol. The α-tocopherol in damaged and destroyed cells is released unchanged or as oxidation products because of *in situ* oxidation during exposure. The released tocopherol and tocopherol oxidation products are transported to the liver where the oxidation products accumulate and are further metabolized to conjugates of α-tocopheronic acid. The fate of the dimer and trimer is unknown.

We could not demonstrate any significant difference in metabolism of retinol acetate between NO_2-exposed and control animals in the one experiment we performed. Because none of the original ^{14}C-retinol acetate and only one spot of radioactive material was recovered from any of the tissues, one possibility is that retinol metabolism is very rapid and that tissue must be examined sooner than 24 hours after injection. However, earlier work indicates that retinol metabolism is not this rapid. Complete recovery of radioactivity from ^{14}C-retinoic acid required 48 hours,[49] and retinyl acetate was metabolized at a nearly constant rate for 6 to 7 days.[50,51] However, these other investigators

administered labeled vitamin A intravenously whereas we used ip administration.

Vitamin A is susceptible to light-catalyzed isomerization and rearrangement as well as to air oxidation. Therefore, another possibility is that the single radioactive spot derived from ^{14}C-retinyl acetate is an artifact of isolation despite our efforts to avoid this circumstance. Thus, the retinol data must be interpreted cautiously. Additional experiments performed under more rigorous conditions are required before we can draw meaningful conclusions about the effect(s) of oxidant gases on vitamin A metabolism.

SECTION VII

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16. ABSTRACT

Rats exposed to atmospheres containing nitrogen dioxide (NO_2) in excess of 10 ppm showed a 50% increase in uptake of ^{14}C-α-tocopherol by the lung when compared with control rats maintained in ambient air. This increase was not observed in liver or blood, the retention of ^{14}C-α-tocopherol being the same in exposed and control animals. NO_2 exposure did not affect the half-life of ^{14}C-α-tocopherol in lung, liver, or blood. The liver of rats exposed to greater than 10 ppm NO_2 or to 1 ppm ozone showed a statistically significant ($P < 0.05$) increase in the level of α-tocopherol oxidation products compared with control rat liver, as judged by an increase in the ratio of α-tocopherol quinone plus α-tocopherol dimer to α-tocopherol. This increase was limited to the liver and was not observed in either lung or blood. Liver, lung, and blood of vitamin E-deficient rats exposed to 5 ppm NO_2 did not show any statistically significant increase in α-tocopherol oxidation products when compared with control tissues. No effect of NO_2 in ^{14}C-retinol acetate metabolism was observed. This research resulted in the first description of an enzyme involved in α-tocopherol metabolism - namely, a UDP-glucuronic acid:dihydro-α-tocopheronolactone glucuronosyl transferase, the final enzyme in α-tocopherol metabolism before excretion. The glucuronosyl transferase is a microsomal enzyme found predominantly in the liver, and does not require a divalent cation for activity, although it is stimulated by Sn^{++}, Ca^{++}, and Mg^{++}.

17. KEY WORDS AND DOCUMENT ANALYSIS

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