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J. BRIAN MUDD

ASSESSING TOXIC EFFECTS OF ENVIRONMENTAL POLLUTANTS

ANN ARBOR SCIENCE

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edited by

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ANN ARBOR SCIENCE
PUBLISHERS INC / THE BUTTERWORTH GROUP
P.O. BOX 1425 ■ ANN ARBOR, MICHIGAN 48106

Second Printing 1980

230 Collingwood, P. O. Box 1425, Ann Arbor, Michigan 48106

Library of Congress Catalog Card No. 78-71430
ISBN 0-250-40266-1

Manufactured in the United States of America

PREFACE

Assessment of chemical toxicity has advanced far from the stages of determining the dose resulting in lethality. Even though this test of lethality provides necessary information, it is important to find other methods of assessment which are rapid and can provide the basis for predicting toxicity. Biophysical and biochemical methods are described in this book which may provide sensitive methods for assessing toxicity as well as discovering the underlying mechanisms by which toxic chemicals act.

The need for information on the toxicity of environmental pollutants is clearly based on the need to protect human health. The first four chapters of this book concentrate on human health, covering research with human subjects and nonhuman primates, and discussing the difficulties of assessing human toxicity on the basis of tests using laboratory animals.

Throughout the book, the emphasis is on the lung, because environmental pollutants are frequently inhaled. Included are discussions of the use of lung organ cultures for assessment of toxicity and the use of specific cell types of the lung, particularly those cells providing resistance to bacterial infection. It is important to know which regions of the lung are affected by inhaled pollutants. One chapter develops methods for predicting these regions based on the chemistry of the pollutant and degree of exercise.

Many pollutants from many sources are examined: primary and secondary pollutants from automobile exhaust, such as carbon monoxide and ozone; pollutants from industrial sources, such as sulfur dioxide; herbicides and pesticides; and cigarette smoke. The effects which are examined range from perturbations of the cellular membrane of the cell to the study of mutagenicity and carcinogenicity.

This book introduces material of interest to people of many disciplines of chemistry and biology who are concerned with deleterious chemicals in our environment. While reviewing progress in these areas of toxicology, new and specific research data are presented. This book should be considered as

a companion volume to *Biochemical Effects of Environmental Pollutants*, S. D. Lee, editor, also published by Ann Arbor Science Publishers, Inc.

The chapters in this book are the outcome of a symposium at the 174th American Chemical Society Meetings in Chicago. The emphasis at the symposium, and in this book, was on the assessment of toxicity of environmental pollutants.

The editors are grateful to Dr. Linda Deans and Dr. Nina McClelland, Chairpersons, Division of Environmental Chemistry, American Chemical Society, for their support of the symposium which led to this book.

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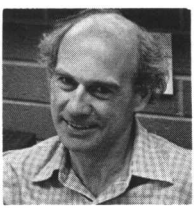
Dr. S. D. Lee is Research Toxicologist, Environmental Criteria and Assessment Office, U.S. Environmental Protection Agency, Cincinnati, and Adjunct Assistant Professor, Department of Environmental Health, University of Cincinnati College of Medicine.

He received his PhD from the University of Maryland, specializing in animal nutrition and biochemistry. He continued his training in biochemistry at Duke University Medical Center under a postdoctoral fellowship from the National Institute of Health. He was awarded an Advance Research Fellowship from the American Heart Association for continuation of his work at Duke.

His research work at EPA has been primarily devoted to the early identification of adverse effects of environmental pollutants using animal models, to obtain necessary information for assessing possible health effects on human populations. More recently, he has been engaged in health assessment and criteria document preparation of various water-associated pollutants.

Dr. Lee is the author of numerous papers presented at national and international conferences and symposia, and has published over forty articles in various professional journals. He is the editor of *Biochemical Effects of Environmental Pollutants*, published by Ann Arbor Science in 1977.



Dr. J. Brian Mudd is Professor of Biochemistry at the University of California, Riverside. He received a Bachelor's Degree from Cambridge University, England, a Master's Degree from the University of Alberta, Canada, and a PhD from the University of Wisconsin. His research has concentrated on the biochemical effects of air pollutants, particularly ozone and peroxyacetylnitrate. This research has ranged from studies of these compounds with biochemicals in well-defined systems to the current studies on cells.

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CHAPTER 1

FROM ANIMALS TO MAN, THE GRAND EXTRAPOLATION OF ENVIRONMENTAL TOXICOLOGY

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INTRODUCTION

There are few areas more important in the application of chemistry than environmental toxicology. To extrapolate from animal experiments to effects on man, a critical understanding of the mechanism of toxicant action is needed. Understanding the chemical mechanism of action allows the proper utilization of animal, plant and microorganism models for studying the potency and effect of a given environmental toxicant. Much of the toxicological data fails to meet this criterion. Much data is simply a repetition of highly standardized and regimented tests of individual compounds to provide an indication of its potency compared to other compounds. While there is no doubt that such screening tests are needed and must be continued, they add little to our knowledge of the overall problem of toxicity. Recent legislative action in the United States leading to the implementation of the Toxic Substances Hazard Act places us in critical shortage of personnel, resources and funds that will not allow us the luxury of examining case by case every compound that is likely to enter the market place or to be dispersed widely in the environment.

It is my view that we must apply the techniques and technology of chemistry to an understanding of the toxicology of classes of compounds and do so on the basis of the molecular chemistry involved. Implicit in

my argument will be the concept that abatement or prevention of exposure is the only safe strategy to control toxicants in the environment and work place. We know of no cure for cancer. We know of no cure for mutagenically related birth defects. Until these modes of therapeutic medicine become available, prevention remains the only cure.

It is the purpose of this chapter to stimulate thought on this problem through a fundamental approach in the hope that by applying this methodology we might predict toxicity. The following are several examples that illustrate both the problems facing the environmental toxicologist and the nature of the chemistry involved.

MUTAGENESIS, CARCINOGENESIS AND METABOLISM

It is proposed that much emphasis be placed on a hierarchical model for testing the very compounds falling under the Toxic Substances Hazard Act. Mutagenesis of such compounds is supposed to be detected by the reversion of specific traits of microorganisms. This test system, widely known as the Ames Test after its originator, Professor Bruce Ames, utilizes a simple bioassay system.[1] Mutants of the microorganism, *Salmonella typhimurium,* have been selected to lack specific traits for growth and survival in medium deficient of specific nutrients. Should the test organism be exposed to a chemical that causes a mutation, by chance some of the mutations will be reversions back to the genome which does not require the externally supplied nutrient for growth. It is then a simple task for the microbiologist to measure the incidence of such mutations by counting the number of revertent colonies after treatment with the chemical in question. The structure of the fire retardant Tris and several decomposition products are shown in Figure 1. Typical data are shown in Figure 2 taken from Dr. Ames' work,[2] in which the number of revertents per plate is directly proportional to the concentration of the potential mutagen that was applied to the test organism. Several decomposition products are not as active as the parent compound. These low mutation rates are typical of many compounds tested. The mutant microorganisms are selected so that they lack any reparative mechanism; thus, the mutation rate detected under these conditions represents the maximum possible. Ames and others have manipulated these and other kinds of microorganisms to reduce the concentration gradient that might exist between the medium and the interior of the microorganism. They have sought to have microorganisms that are freely permeable to a large number of complex organic compounds.

Often the chemical is not mutagenic in itself but requires metabolism to a transient but highly reactive intermediary, which appears to bind

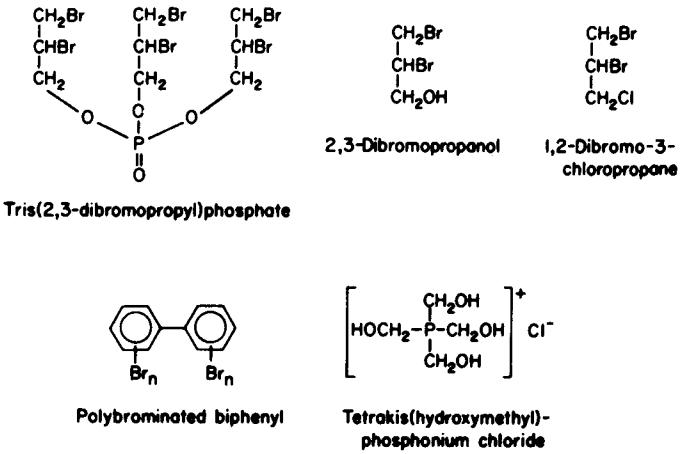


Figure 1. Chemical structure of the fire retardants and related compounds. (From Blum and Ames,[2] with permission of the authors and publishers.)

covalently to the nucleic acid or the macromolecules of the test organism. Activation in this system is accomplished by the addition of a crude microsomal preparation obtained from the liver of some mammal. Much of the activation appears to be carried out by the mixed function oxidases of the hepatic microsomal fraction called S9. NADPH and molecular oxygen are required. In the Ames system then, NADPH or an NADPH generating system is added to the microsomal fraction along with the test organism and the toxicant. The results in Figure 2 are in the presence of this fraction isolated from the liver of a rat induced by prior treatment with Arochlor.

A major unresolved question in the use of the Ames method of activation of foreign compounds is the chemical nature of the products formed. The chemical composition of most of the activated compounds found to be mutagenic has been inferred by the use of preformed metabolites thought to be the ultimate product of the microsomal oxidase. Arene oxides have been proposed as the most common intermediaries for polycyclic aromatic hydrocarbon-type compounds. Alkylation is assumed to be the most common mechanism of reaction between the intermediary toxicant and DNA. Until the chemical nature of the activated compounds reaching the microorganism is known, one cannot be sure of the ultimate utility of this test in terms of extrapolation to man. Purposefully, the Ames test has been rendered more sensitive by eliminating the soluble enzymes capable of detoxification of the "activated" metabolites formed

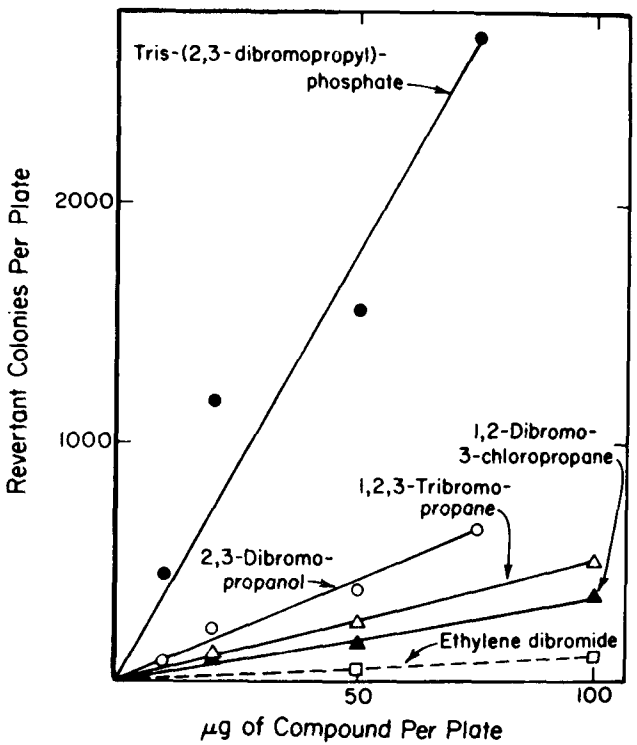


Figure 2. Number of revertant mutations produced in *Salmonella* strain TA100 by the fire retardant Tris and related compounds. The number of revertant colonies per plate is a measure of the number of mutations. Revertant colonies represent bacteria capable of growing in the histidine-deficient medium. Ethylene dibromide was added to the plates at 10 times the scale values. The results with tris-BP, 2,3-dibromopropanol and dibromochloropropane were obtained in the presence of the activating system from an Arocholor-treated rat. (From Blum and Ames,[2] with permission of the authors and publishers.)

by the microsomal oxidase. Little progress has been reported in this area, so one cannot be sure that a compound found to be mutagenic in the Ames system would be mutagenic in man. It may be rapidly detoxified and not have an opportunity to express its mutagenic potential. The kinetics of conversion and detoxification have not been fully worked out for model compounds, as is evident in the following discussion on the non-mutagenic toxic effects of covalent binding.

It is also clear that multiple forms exist[3] of the principal microsomal oxidase, cytochrome P_{450}. The expression of these isoenzymes depends on both the genome of the animal donating the microsomes and the environmental exposure of the animal. Sometimes, inducers of cytochrome P_{450}

are used prior to the preparation of the liver S9 fraction in the hope of increasing the activity of the preparation and, therefore, the sensitivity of the test. Since the products of the monooxygenase remain elusive, one cannot be sure of the chemical effect resulting from the use of an S9 fraction containing different isomeric forms of cytochrome P_{450}. Again, much progress can be gained from the chemical identification of the products formed using these different isoenzymic cytochrome P_{450} preparations.

The chemical nature of the reaction between the target within the microorganism and the "activated" mutagen is also not clearly understood; nor, for that matter, is the target macromolecule fully known. In more complex eukaryotic cells, reaction with macromolecules other than nucleic acids may result in mutagenesis through complex mechanisms such as reverse transcriptase or alterations in local membrane properties. False negative tests could result from such imperfections in the microorganism-based test.

A major problem exists in the question of a "threshold" or "no effect" dose for mutagens. Certainly, the concentration and frequency of naturally occurring mutagens and carcinogens in plants and foods are greater than those thought to occur from the apparent mutation and cancer rates in man. Since we do not know the pharmacokinetics of the absorption, distribution, activation and elimination of these mutagens, one cannot presume an inherent ability of man to repair mutagenesis at certain low doses of mutagens or carcinogens. The existence of a "no effect" dose for a given carcinogen may be due to an artifact of the kinetics of these reactions unique to that carcinogen and not to a general phenomenon.

Lastly, the heart of the hypothesis that carcinogenesis is due to mutagenesis remains to be proved. One must admit that the prediction thus far by microorganism-based tests is impressive and that all carcinogens have proved to be mutagens. Not all mutagens have proved to be carcinogens, however. Quite properly, this fact has led to the development of a hierarchical approach, wherein the microbiological test is the first tier. Unfortunately, there is a growing tendency to stop at the Ames test once the compound has been found mutagenic. Likewise, the Ames test does not predict any other toxic reactions. The pulmonary toxicity of the herbicide paraquat could not be predicted from the Ames test and represents to the occupationally exposed as great a hazard as a potential malignancy.

Metabolic activation to reactive intermediaries, which combine covalently with cell constituents, is a general toxic reaction not confined to mutagens or carcinogens. The pioneering work[4] in demonstrating that drugs and other toxicants may react covalently through activation by a microsomal system with tissue constituents *in vivo* is illustrated with acetaminophen.

Acetaminophen induces hepatic necrosis when given above a critical concentration. The time course of covalent binding of tritiated acetaminophen to mouse tissues has been measured by Jollow, *et al.*[3] Covalent binding of acetaminophen to the liver occurs rapidly while muscle tissue has very little, if any, binding. Acetaminophen is then rapidly metabolized in the liver to an intermediary covalently bound to tissue macromolecules, which is only slowly removed during 24 hours after the administration. The maximum covalent binding of acetaminophen under these studies, which is typical of the metabolism of drugs that bind covalently to the liver, is only about 2 nmol/mg of tissue protein. In the conventional balance table approach to studies of distribution and uptake of drugs and toxicants, such binding would represent only a miniscule amount, less than 0.01% of the total dose given. The detection of such amounts is unlikely without high specific activity radiolabeled compounds.

Activation of compounds leading to tissue alkylation is presumed to proceed via arene oxides. Other pathways that prevent tissue alkylation by detoxification, such as hydration and rearrangement, exist in mammalian tissues. Conjugates with glutathione may or may not represent full detoxification. Glutathione is a good leaving group, and subsequent displacement reactions on the conjugate can occur.

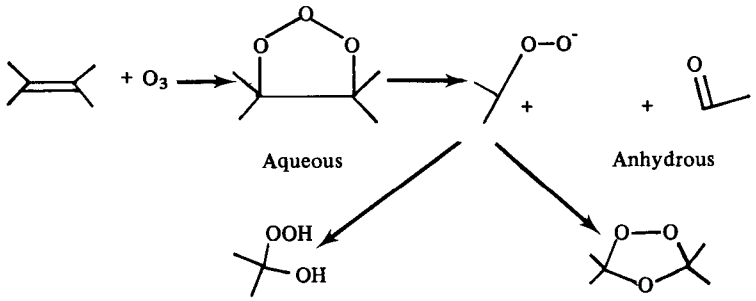
A critical problem is to model this more complex balance of activating and detoxifying reactions in a system such as the Ames test. At present, we can only presume that any mutagen detected by the microbial system is a potential carcinogen.

Mutation need not lead to malignant transformation. Mutation could lead to a selective advantage on the part of the daughter cells, promoting a benign growth. The monoclonal nature of atherosclerotic plaques has led to the suggestion that this chronic disease might be due to a single mutation in the fibrocytes lining the arteries.[5] Other chronic diseases having their expression years after the initiating event could also be the result of a single mutation, which is propagated slowly by the development of clones within an organ system. Degenerative diseases of a monoclonal nature may then be caused in part by environmental mutagens.

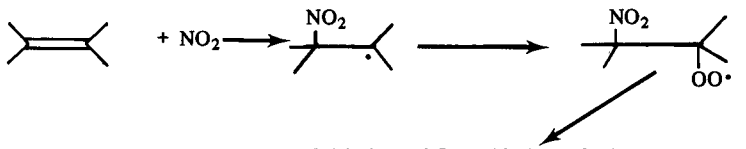
HORMONAL PATHWAYS OF TOXICITY

Air pollutants react with tissue constituents and thereby may produce "ultimate toxicants" in a manner analogous to mutagenic and carcinogenic materials. Ozone and nitrogen dioxide react readily with a variety of model molecular species to produce reactive compounds.

Unsaturated fatty acids are particularly susceptible to attack yielding peroxides, ozonides and aldehydes, which have systemic effects in organs other than the lung.



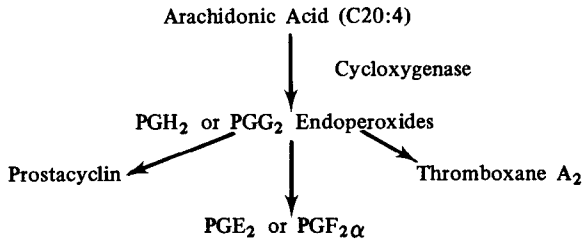
Initiation of Peroxidation
of Tissue Unsaturated Lipids



Initiation of Peroxidation of Tissue
Unsaturated Lipids

Dietary effects also occur. Vitamin E deficiency increases the toxicity of ozone and nitrogen dioxide.[6] For instance, sleeping time of mice due to pentobarbital injection is elongated by prior exposure to ozone, suggesting a direct effect on the liver.[7]

Hormonal-like activity is produced by ozone-catalyzed autoxidation of arachidonic acid similar to the enzyme-catalyzed oxygenation, which is the initial step in the formation of prostaglandin and related hormones.



Hormonal activity, similar to prostaglandin endoperoxides PGG_2 and PGH_2, has been found using assays with human platelets.[8] Human platelets are rapidly aggregated by arachidonic acid endoperoxides formed during the ozone autoxidation of arachidonic acid. Aggregation is indicated in Figure 3 by the downward deflection due to a change in

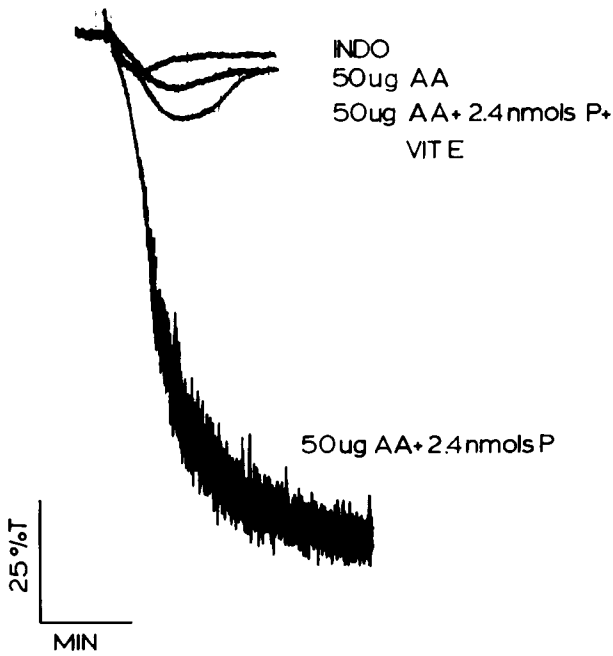


Figure 3. Aggregation of human platelets by arachidonic acid peroxides illustrating the prostaglandin-hormone-like activity. Arachidonic acid (AA) or AA with vitamin E ($1:30^3$ molar ratio of AA to vitamin E) was peroxidized by a stream of 2.0 ppm ozone for 1 minute. Platelets were aggregated by AA, arachidonic acid peroxides (AAP) formed with the presence or absence of vitamin E. Platelets were or were not preincubated with 14 μM indomethacin (Indo), an inhibitor of platelet cycloxygenase. (From Roycroft, *et al.*,[8] with permission of the authors and publishers.)

turbidity of a suspension in human platelet-rich plasma. Vitamin E, a required nutrient for man and animals, has a profound effect on the biological potency of the peroxides formed. Vitamin E abolishes most of the biological activity while not inhibiting the formation of peroxides. The ozone-formed peroxides also contracted aortic spiral strips and fundus strips, demonstrating biological activity on smooth muscles nearly as potent as the natural endoperoxides, PGG_2 and PGH_2.

A scheme for the formation of cyclic peroxides during the autoxidation of cellular unsaturated fatty acids is shown in Figure 4. Toxicity through lipid peroxidation begins with the abstraction of a hydrogen from the *cis*-methylene-interrupted fatty acids found in all cells. Fatty acids having

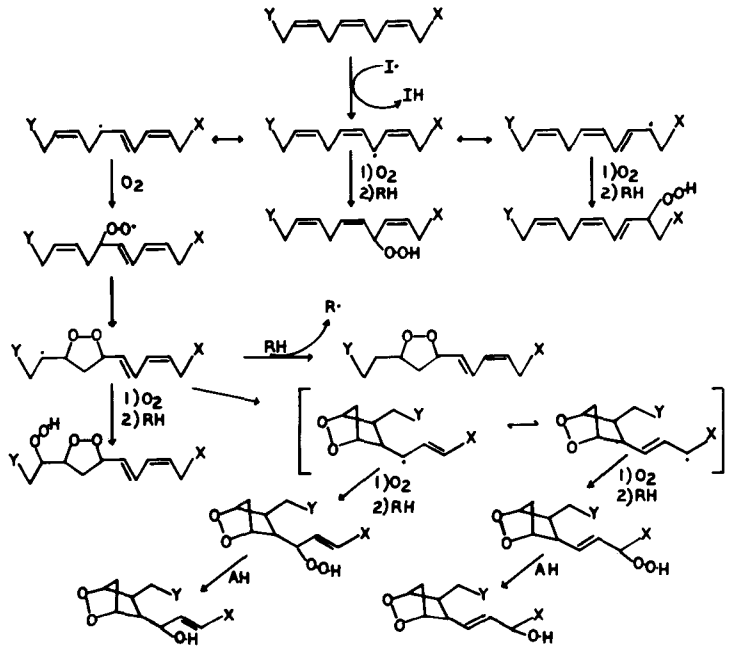


Figure 4. A scheme for the formation of cyclic peroxides having hormone-like activity on the peroxidation of unsaturated fatty acids. X and Y represent the carboxylic and alkyl residues of the fatty acid. Peroxidation is initiated by a free radical I · Hydrogen abstraction from another fatty acid molecule RH provides another radical R· for propagation of the reaction. (From D. B. Menzel, "Environmental Toxicants and Vitamin E" in *Vitamin E,* L. Machlin, Ed., (New York: Marcel Dekker Press, (In Press), with permission of the publishers.)

three or more unsaturations are of particular interest. One resonance form can occur that will result in the formation of a peroxyl free radical β-γ to an unsaturation. Such a peroxyl group can cyclize to form an endoperoxide. This reaction path is thought to be that of the enzyme cycloxygenase, which forms PGH_2 and PGG_2 from arachidonic acid. Prostanoic acid can result from such a cyclization and has been isolated from peroxidizing polyunsaturated fatty acids. Nonenzymatic and enzymatic conversion of unsaturated fatty acids having greater than three unsaturations, and hence the possibility of a β-γ unsaturated peroxyl free radical, results in the same prostaglandin product. Under this scheme, the end effect is independent of the nature of the initiating compound. Peroxidation could come from inhaled ozone (O_3) and nitrogen dioxide (NO_2) or from the metabolic activation of ethanol or carbon tetrachloride. All of the exposures