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DNA Adduct Formation by Twelve Chemicals with Populations Potentially Suitable for Molecular Epidemiological Studies

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Printed in the United States of America. Available from
National Technical Information Service
U.S. Department of Commerce
5285 Port Royal Road, Springfield, Virginia 22161
NTIS price codes—Printed Copy: A07; Microfiche A01

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ORNL/TM-10849

Health and Safety Research Division

DNA ADDUCT FORMATION BY TWELVE CHEMICALS WITH POPULATIONS POTENTIALLY SUITABLE FOR MOLECULAR EPIDEMIOLOGICAL STUDIES

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February 1989

Supported by
Health Effects Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, NC under
Interagency Agreement No. 1739-1739-A1;
EPA No. 89931767-01-1

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Prepared by the
OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37831
operated by
MARTIN MARIETTA ENERGY SYSTEMS, INC.
for the
U.S. DEPARTMENT OF ENERGY
under Contract No. DE-AC05-84OR21400

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CONTENTS

TABLES vii

1. EXECUTIVE SUMMARY 1

1.1 INTRODUCTION 1
1.2 GENERAL CONCLUSIONS 5
1.3 INDIVIDUAL CHEMICALS 6
1.3.1 Ethylene Oxide 6
1.3.2 Styrene and Styrene Oxide 7
1.3.3 Vinyl Chloride 7
1.3.4 Epichlorohydrin 8
1.3.5 Propylene Oxide 8
1.3.6 4,4' -Methylenebis-2-Chloroaniline 9
1.3.7 Direct Blue 6, Direct Black 38, Direct Brown 95, and Benzidine 9
1.3.8 Acrylonitrile 10
1.3.9 Benzyl Chloride 10
1.4 CONFOUNDING FACTORS 11

2. ETHYLENE OXIDE 13
2.1 CHEMISTRY 13
2.2 ABSORPTION 13
2.3 METABOLISM 14
2.1.1 Half-Life 14
2.3.2 Conversion of Ethene to EO 14
2.3.3 Urinary Metabolites of Ethylene Oxide 15
2.4 DNA ADDUCT FORMATION 15
2.4.1 DNA Adduct Formation 15
2.4.2 Observed Binding of EO to Hb and Other Proteins 17
2.4.3 Human Dosimetry 19
2.5 DNA DAMAGE STUDIES 20

3. STYRENE 23
3.1 CHEMISTRY 23
3.2 ABSORPTION 23
3.3 METABOLISM 25
3.4 DNA ADDUCTS 28
3.5 PROTEIN ADDUCTS 31
3.6 DNA DAMAGE 31

4. VINYL CHLORIDE 33
4.1 METABOLISM 33
4.2 ADDUCT INFORMATION 35
4.2.1 *In Vivo* and *In Vitro* DNA and RNA Adducts 35
4.2.2 *In Vitro* Modification of Bases 38
4.3 STRUCTURE ACTIVITY RELATIONSHIPS 38

4.4 BIOLOGICAL ENDPOINTS 40
4.4.1 VC-Induced Base-Pair Miscoding 40
4.4.2 Carcinogenic Potency from DNA Adduct Levels .. 40

5. EPICHLOROHYDRIN 43
5.1 CHEMISTRY 43
5.2 METABOLISM 43
5.3 DNA ADDUCT FORMATION 44
5.3.1 *In Vitro* DNA Adduct Formation 44
5.3.2 DNA Damage Assays 45
5.3.3 Estimation of Risk to Humans 45

6. PROPYLENE OXIDE 47
6.1 CHEMISTRY 47
6.2 ABSORPTION 48
6.3 METABOLISM 48
6.4 DNA ADDUCT FORMATION 48
6.4.1 *In Vitro* DNA Adduct Formation 48
6.4.2 Observed Binding of PO to DNA and Hb *In Vivo* 48

7. 4,'4-METHYLENEBIS-2-CHLOROANILINE 51
7.1 CHEMISTRY 51
7.2 METABOLISM 51
7.2.1 Human Metabolism 51
7.2.2 Rat Metabolism 52
7.2.3 Dog Metabolism 52
7.3 DNA ADDUCT FORMATION 52
7.4 DNA DAMAGE STUDIES 53

8. BENZIDINE AND BENZIDINE DYES: DIRECT BLUE 6, DIRECT BLACK 38, AND DIRECT BROWN 95 55
8.1 CHEMISTRY 55
8.2 METABOLISM 56
8.2.1 Metabolism of the Dyes 56
8.2.2 Metabolism of Benzidine 56
8.3 DNA ADDUCT FORMATION 58
8.3.1 Adducts Induced by Benzidine Dyes 58
8.3.2 Benzidine-Derived Adducts 59
8.4 DNA DAMAGE STUDIES 61

9. ACRYLONITRILE 63
9.1 CHEMISTRY 63
9.2 METABOLISM 63
9.2.1 *In Vitro* Microsome/Acrylonitrile Interactions 63
9.2.2 *In Vivo* Metabolism Studies 64
9.2.3 Urinary Metabolites 64
9.2.4 2-Cyanoethylene Oxide 65
9.3 MACROMOLECULAR ADDUCT FORMATION 66
9.3.1 *In Vivo* DNA Adducts 66
9.3.2 *In Vitro* Binding to DNA and RNA 67

9.3.3 *In Vitro* Nucleoside Studies 67
9.3.4 *In Vitro* Studies with Polynucleotides 67
9.3.5 *In Vitro* DNA Studies 68
9.3.6 DNA Adducts in Common with Other Chemicals ... 69
9.3.7 Binding to Proteins 69

9.4 DNA DAMAGE STUDIES 69
9.5 *IN VITRO* TRANSFORMATION 70

10. BENZYL CHLORIDE 71
10.1 CHEMISTRY 71
10.2 ABSORPTION 71
10.3 METABOLISM 72
10.4 DNA ADDUCT FORMATION 72
10.4.1 *In Vitro* Studies with Bases 72
10.4.2 Observed Binding of Benzyl Chloride to DNA and Hb 73
10.4.3 Similar Adduct Formation by Other Chemicals 74

11. ANALYTICAL METHODS 75
11.1 INDIVIDUAL CHEMICALS 75
11.1.1 Ethylene Oxide 75
11.1.2 Styrene 75
11.1.3 Vinyl Chloride 75
11.1.4 Epichlorohydrin 76
11.1.5 Propylene Oxide 76
11.1.6 MOCA 76
11.1.7 Benzidine Dyes and Benzidine 77
11.1.8 Acrylonitrile 77
11.1.9 Benzyl Chloride 77
11.2 GENERAL DISCUSSION ON ANALYTICAL METHODS 78

12. RESEARCH NEEDS 81

REFERENCES 85

APPENDIX A A-1
A.1 ESTIMATION OF DOSE FROM DNA OR Hb ALKYLATION A-1
A.2 USE OF THE PROPORTIONALITY RELATIONSHIP TO EVALUATE THE RELATIVE BIOLOGICALLY EFFECTIVE DOSE ... A-2
A.3 APPLICATION OF ADDUCT FORMATION TO EXPOSURE MONITORING A-2
A.4 CORRELATION OF EXPOSURE TO RISK A-3
A.5 RISK ESTIMATION FROM LEVELS OF ADDUCTS A-5
REFERENCES A-6

TABLES

1.1 Chemicals reviewed and *in vivo* adduct formed 2

2.1 DNA adducts formed from ethylene oxide 16

2.2 Tissue levels of protein adducts in CBA male mice 18

2.3 Hemoglobin adducts 20

3.1 Constants for absorption of styrene by humans 24

3.2 *In vivo* adducts derived from styrene and styrene oxide ... 29

3.3 DNA adducts formed from styrene oxide 30

3.4 Deoxynucleoside reaction products with styrene oxide 30

4.1 Binding of CEO precursors to macromolecules *in vivo* 34

4.2 Chemical sources of VC-related adducts 35

4.3 *In vivo* alkylation of DNA in rodents by VC 36

4.4 Half-lives of DNA adducts derived from vinyl chloride exposure 37

4.5 *In vitro* synthesis of VC-related adducts 39

5.1 Adducts formed from epichlorohydrin 44

5.2 *In vivo* binding of radioactive ECH to rat and mouse organs 45

6.1 Propylene oxide adducts 49

8.1 Rates of N-oxidation of acetylated BZ metabolites by mouse and rat liver microsomes 58

8.2 Persistence of DNA adducts 59

8.3 DNA adducts formed from benzidine and benzidine dyes, and benzidine derivatives 60

9.1 Adducts formed from acrylonitrile *in vivo* 66

9.2 Adduct derivatives formed from acrylonitrile *in vitro* 68

12.1 Potential concomitant exposures in otherwise suitable population group 82

12.2 Chemicals that give rise to the same adduct(s) 83

EXECUTIVE SUMMARY

1.1 INTRODUCTION

This review summarizes macromolecular adduct information available through October 1987 for evaluating the feasibility of designing an adduct-based molecular epidemiological study. Such a study ideally would examine an excess exposure to a single chemical and would use DNA adduct formation as a bioindicator of exposure. The feasibility of an epidemiological study using molecular biomarkers or adducts as quantitative measures of exposure and predictors of adverse health effects depends on four general variables: (1) how adducts are formed and whether they are stable long enough to be detected; (2) the degree of understanding of the relationship between the biomarker and the specific biological response of concern (e.g., carcinogenesis, mutation, etc.); (3) the measurability of the biomarker with relatively noninvasive sampling techniques; and (4) the availability of human populations with sufficient size and uniqueness to provide an acceptable level of uncertainty in the data analysis.

This manuscript focuses on the first two variables and includes some examination of the third, the measurability of the adducts formed. No discussion of the last variable is included, as population availability was one of the primary criteria for selection of the chemicals shown in Table 1.1 for review. Selection of the chemicals--eight individual chemicals and a group of three benzidine-related chemicals--was made by comparing potential study population quality and availability (Zeighami et al. 1987 a,b) with DNA adduct information from two lists (94 chemicals reported to form DNA adducts and 283 chemicals taken from the EPA/HERL genotoxicity list [Waters, Stack, and Brady 1987]). A novel approach to population analysis was used to put priorities on the chemicals for review (Zeighami and Griffith, in preparation).

The present review and the analysis of population availability were conducted as the first steps in developing an optimal basis for molecular epidemiology programs. The purpose of such programs is to improve the ability to assess health risks resulting from exposure to toxic chemicals. One facet of this program would be to relate, if possible, the presence of DNA adducts in body fluids or tissues of a human population to a particular chemical exposure in a quantitative way and also to relate a particular health effect, or toxic endpoint, to the adduct level and exposure. Another is to relate the human exposure/adduct formation/disease endpoint information to exposure/adduct formation/disease or biological endpoint information from whole animal and *in vitro* screening assays to improve the quantitative predictive capability of such procedures. The rationale for these approaches is as follows.

Epidemiological studies of changes in disease risk induced by exposure to an environmental agent are invariably handicapped by uncertainty in the degree and duration of exposure in the human population under study. Since the exposure may have occurred many years before the expression of the measurable event, exposure estimates in

Table 1.1. Chemicals reviewed and *in vivo* adducts formed

Chemicals	*In vivo* adducts
Ethylene oxide (75-21-8)[a]	N^7-(2-hydroxyethyl)-guanine O^6-(2-hydroxyethyl)-guanine
Styrene (100-42-5)	N^7-(α,β)(7(8)-hydroxyethylphenyl)-guanine N^2-(α,β)(7(8)-hydroxyethylphenyl)-guanine
Vinyl chloride (75-01-04)	N^7-(2-oxoethyl)-guanine N^2,3-ethenoguanine
Epichlorohydrin (107-07-3)	Unidentified
Propylene oxide (75-56-9)	Unidentified
MOCA (101-14-4)	Unidentified
Direct Black 38 (1937-37-7)	Unidentified
Direct Blue 6 (2602-46-2)	N-(deoxyguanosin-8-yl)-N'-acetyl-benzidine N-(deoxyguanosin-8-yl)-N'-benzidine Unidentified larger-sized adduct
Direct Brown 95 (16071-86-6)	Unidentified
Benzidine (92-87-5)	N-(deoxyguanosin-8-yl)-N'-acetyl-benzidine N-(deoxyguanosin-8-yl)-N,N'-diacetyl-benzidine
Acrylonitrile (107-13-1)	N^7-(2-oxoethyl)-guanine
Benzyl chloride (100-44-7)	N^7-(7-benzyl)-guanine

[a]Chemical Abstracts Service number.

humans are generally limited to indirect reconstructions of exposure likelihood. When exposure estimates are possible, the degree to which exposure level reflects actual tissue dose is unknown. Further, most human exposures, both in the occupational setting and the environmental setting, are known to be to complex mixtures of materials. The attempt to link an effect to a single exposure or to quantify the degree of risk resulting from exposure to a single material is either difficult or impossible. The availability of individual, as opposed to group, measurements of human exposure to a potential carcinogen may prove critical for establishing a causal relationship between the agent and a specific disease endpoint and for improving the linkage between exposure and risk. The purer the exposure and the more individualized the exposure measurement, ideally at the level of target cells, the stronger is the basis on which inferences of causality can be built. Measurements of specific biological parameters, including determinations of carcinogens and/or their cellular macromolecular adducts, may thus strengthen inferences from epidemiological studies by individualizing the characterization of exposure in two ways: (1) by allowing the measurements to be made at the individual (tissue and body fluids) rather than group level; and (2) by making the measurements specific for a single chemical. Obviously, in practice, the success of this approach will be strongly dependent on the stability of the biological parameter to be measured and on its ability to reflect acute and/or chronic, present and/or past exposures, and the development of adequately sensitive assays.

The ability to measure chemically-induced DNA damage in specific tissues is important in risk assessment because alteration of the DNA appears to be a key step in the initiation of carcinogenesis. Alteration of the daughter DNA sequence either by error-prone repair or deletions is the accepted mechanism that perpetuates the chemical damage. These permanent changes to the genome (genotoxicity) may then affect the normal differentiation processes so that subsequent generations of cells exhibit aberrant growth patterns leading to carcinogenesis and other abnormalities.

A number of short-term tests have been used to quantify the extent of genotoxicity using mammalian or bacterial cell culture assays. For many classes of chemicals, correlations exist between genotoxicity in the short-term tests and animal carcinogenicity. These *in vitro* systems are, however, severely limited in their predictive abilities because they often do not reflect the important biological parameters of species, strain, sex, and organ specificities commonly observed in chemical carcinogenesis. Unfortunately, precise methods for extrapolating the results obtained from animal bioassays and *in vitro* tests to humans are still unavailable, as is the fundamental knowledge of the circumstances under which such extrapolation provides legitimate estimates of risk for humans.

In view of these difficulties, the study of risks to human populations from environmental exposures requires a methodology that minimizes biological uncertainty by combining epidemiological techniques with laboratory techniques that measure specific biochemical and

molecular parameters in human tissues and biologic fluids. A molecular marker with great potential as an indicator of the effective biological dose of an environmental carcinogen is the covalently bound adduct formed between the substance, or its metabolite, and DNA. Covalent binding assays have demonstrated that a number of carcinogens are metabolically activated to electrophilic species capable of reacting with electron-rich atoms in cellular DNA. The covalent binding of a material to cellular DNA appears to be a critical event in the carcinogenic process.

Advantages of monitoring adduct formation by specific chemicals include the facts that:

* it takes into account absorption by all routes,
* it integrates exposure from all sources,
* it can be used as a basis for estimation of risk from multiple chemicals, and
* data obtained may be directly related to adverse effects, and thus may provide a better estimate of risk than does monitoring average levels of chemicals in the environment.

However, there has been only limited work to date on the use of DNA adduct detection for the estimation of exposure in human populations exposed to multiple sources and by multiple routes over a long period of time. Ultimately, the utility of any biomonitoring technique, including the detection of DNA adducts for use in a human population, will depend on the adducts' patterns of formation and decay in the body and the development of rapid, simple, noninvasive screening techniques for the measurement of the material in body fluids or tissues. In this regard, sophisticated radioactive technologies have been developed to detect adduct levels of one per genome. Recently there has also been great interest in developing new, nonradioactive techniques for monitoring bioindicators such as DNA adducts.

Since DNA damage and, in many cases, the formation of stable carcinogen-DNA products are considered critical events in the initiation of the carcinogenic process, it seems likely that assays for the level of carcinogen-DNA adducts in target cells might provide a measure of the biologically effective dose for certain carcinogens. Coupling the human studies with *in vivo* and *in vitro* screening assays will not only provide the means for refining measurement techniques for biomarkers and for putting priorities on the human samples for analysis, but should also provide data useful for extrapolating with more confidence from *in vitro* assays and whole animal studies to human health risk.

Subsequent steps in a molecular epidemiology program then, after the study reported in this document, would include the development and testing of protocols for obtaining human tissues or body fluid samples, development or adaptation and refinement of measurement techniques for the chosen biomarkers to ensure adequate specificity and sensitivity, detailed evaluation of the information on genotoxicity of the selected chemicals, and finally, design of additional *in vitro* and animal studies

to define further, as necessary, the relationships between exposure and biomarker formation and biomarker and toxic biological response.

1.2 GENERAL CONCLUSIONS

This executive summary documents the key results of the detailed review, both general conclusions and chemical-specific information. The general conclusions are as follows:

* Review of most of the papers on which this manuscript is based suggests that DNA adducts would be useful measures of exposure.

* Much evidence indicates that identifying and quantitating the levels of DNA adducts would be valuable in predicting excess risk of developing latent diseases such as cancer or of incurring adverse reproductive effects.

* Ethylene oxide, styrene, vinyl chloride, Direct Blue 6, and benzyl chloride have known DNA adducts isolated from *in vivo* exposure.

* Of these, styrene and benzyl chloride have unique adducts that have not been observed as adducts of other chemicals. Direct Blue 6 also gives a unique adduct among those identified to date, but it is possible that other benzidine dyes may also give rise to that adduct.

* While only five of the chemicals have identified adducts, the chemistry of the remaining chemicals suggests that adduct formation is feasible. Thus each of the chemicals could be used for epidemiological field studies after identification of adducts and of specific populations and after development, or adaptation and refinement, of detection methods.

* Physical constants can be derived from adduct formation studies that may be used in reducing uncertainty in risk calculations. An example would be the rate of reaction of DNA with the reactive form of a chemical. Such values are available for some of the reviewed chemicals.

* The sensitivity of the detection methods currently used for all the chemicals reviewed should be further improved for monitoring DNA adduct levels under conditions of low exposure.

* New methods exist that have the potential for adaptation and development to provide the needed sensitivity and specificity for monitoring low levels of DNA adducts for the chemicals of interest.

* Criteria for selection of the tissue to be monitored must be developed.

* Two types of sampling problems need to be addressed. First, the number and type of measurements needed must be determined. Second, protocols must be worked out for obtaining and handling of samples prior to adduct analysis.

* Mathematical tools for using DNA adduct information in a predictive way need development.

1.3 INDIVIDUAL CHEMICALS

The following chemicals are reviewed in depth in the report: ethylene oxide; styrene; vinyl chloride; epichlorohydrin; propylene oxide; 4,4'-methylenebis-2-chloroaniline (MOCA); the benzidine dyes, Direct Black 6, Direct Black 38, and Direct Brown 95; acrylonitrile; and benzyl chloride. A brief summary for each chemical follows.

1.3.1 Ethylene Oxide

Ethylene oxide (EO) is a known carcinogen, mutagen, and teratogen in animals and is considered a suspect carcinogen in humans. It may also be associated with excess spontaneous abortion in humans. More information is available on DNA adduct formation, persistence, and relation to biological responses for EO than for any of the other reviewed chemicals. Ethylene oxide reacts directly with DNA without metabolic "activation" (transformation to a chemically active metabolite). Ethylene oxide is also highly reactive *in vivo* with a detoxification - excretion half-life of 9 min. It mimics radiation in its physical penetration of tissue. N^7-(2-hydroxyethyl)-guanine, abbreviated as N^7-(2-HOEt)-Gua, constitutes 90% of total EO adducts. This adduct has a removal (repair) half-life in several tissues of 20-32 h depending on the sampling time. Several minor adducts have also been identified. Ehrenberg and coworkers have used a radiation dosimetry model to develop a model for estimating both exposure and risk from observed levels of EO. Calculations based on this model give the unit dose of EO (1 ppm·h) a risk equivalent to 10 mrad-equiv. This approach is described in more detail in the section of the review on EO.

There is some question as to whether N^7-(2-HOEt)-Gua can be related to a meaningful biological response. It may be a useful measure of exposure and of cytotoxicity, but studies suggest it may not give rise to significant mutations. There is not enough information on one of the

minor EO adducts, O^6-(2-HOEt)-Gua, to evaluate its rates of formation and destruction or its potential as a practical biomarker of exposure or latent disease.

The possible use of hemoglobin (Hb) adduct formation by EO as a surrogate for DNA adduct formation has been studied by Ehrenberg and coworkers. Exposure estimates deduced from Hb adduct formation have greater correspondence to those from DNA adduct studies under conditions of continuous exposure in which the steady-state level of Hb adduct formation is considered to be proportional to the average red cell lifetime exposure. While this relationship has been demonstrated in rodents under well-controlled experimental conditions, no experimental data were found that used irregular exposures as would be encountered in real life. The use of Hb adducts as surrogates for DNA adducts from EO exposure is complicated by background levels of indirect EO exposure from inhalation of cigarette smoke (ethene from smoke is metabolized to EO) and by the fact that at least two other constituents of cigarette smoke give rise to N^7-(2-HOEt)-Gua.

1.3.2 Styrene and Styrene Oxide

Styrene and its metabolite, styrene oxide, are likely animal carcinogens. There is limited evidence for reproductive effects in humans, but substantial evidence exists for cellular mutagenicity and chromosomal damage. Considerable information is available on the *in vivo* absorption and metabolism of styrene. DNA adducts of styrene and styrene oxide have been identified. Styrene oxide, a potent direct-acting carcinogen in animals, can be formed from styrene by two independent enzyme systems. Styrene, styrene oxide, and styrene glycol have been measured in human blood during a work day.

The only identified DNA adducts formed *in vivo* from styrene are the N^7-(7(8)-hydroxy-8(7)-phenylethyl)guanine(α,β). These unique structures were formed at relatively high doses (1.1 to 4.9 mmole/kg bw). Reaction of styrene oxide with deoxynucleosides and DNA *in vitro* gives rise to adducts with all four of the bases including the isomers found *in vivo*. Identification of the genotoxic adduct(s) has not yet been made.

1.3.3 Vinyl Chloride

Vinyl chloride (VC) induces human cancers in the lung, brain, and liver, including the rare hemangiosarcoma. Chemistry, metabolism, and DNA adduct formation have received significant study. Vinyl chloride requires activation through epoxide formation to chlorethylene oxide (CEO) which can spontaneously rearrange to chloroacetaldehyde (CAA). While most of the available evidence indicates that CAA preferentially modifies proteins, CAA has been found to form the putative genotoxic adduct, 3,N^2-ethenodeoxyguanosine (2,3-EG). The major adduct derived from VC, N^7-(2-oxoethyl)guanine (7-OEG), has also been identified after *in vivo* exposure. The genotoxicity of the two *in vivo* DNA adducts has been studied, and only the 2,3-EG adduct interferes with a transcription assay using reverse transcriptase. The more abundant DNA adduct, 7-OEG,

does not cause miscoding in a replication fidelity assay and is considered not to be mutagenic. Furthermore, the minor adduct, 2,3-EG, was not found in DNA from adult rats but was found in DNA from 12-d old rats that are more susceptible to VC-induced carcinogenesis than are adult rats. These observations suggest that 2,3-EG is a good candidate as a unique biomarker for the study of latent health effects.

Radioactive tracer studies have been used to characterize VC adduct formation except for two cyclic etheno-adducts formed *in vitro* that are detectable using fluorescence methods. The relevance of these adducts to *in vivo* exposure determination is questionable as they have not been observed in *in vivo* studies.

1.3.4 Epichlorohydrin

Evidence for epichlorohydrin (ECH)-induced disease is limited. Indications have been reported for tumor formation at the site of injection, lung papillomas, and for epidemiological evidence for increased incidence of birth defects. Epichlorohydrin is a direct-acting alkylating agent. It has a lifetime of 20 min *in vivo* compared to a half-life of 1 h for hydrolysis at 37°C in 0.1 M HCl. The metabolites have longer lifetimes; for example, the hydrolytic open-ring product α-chlorohydrin (ACH) has a lifetime of about 5 h.

The binding of ECH to rat (mouse) liver DNA is significant at 0.2 (0.14) pmol adduct per 10^6 pmol bases. No specific evidence has been presented on the structure of the bound adduct, but the high level of single strand breaks in DNA suggests coupling of the ECH to the phosphate diesters. The reaction of ECH with DNA *in vitro* results in the formation of N^7-(±)(3-chloro-1-hydroxy-2-propyl)guanine and N^6-(±)(3-chloro-1-hydroxy-2-propyl)-deoxyadenosine. Ehrenberg and Hussain calculate a unit dose of ECH (1 ppm·h) to have a risk corresponding to about 25 mrad-equiv. A possible alternate source of the same adduct exists in that ECH is also believed to be formed by metabolic dehydrohalogenation of 1,2-dibromo-3-chloropropane.

1.3.5 Propylene Oxide

Propylene oxide (PO) is mutagenic to yeast, bacteria, phage, and fruit flies and induces chromosome damage in human lymphocytes *in vitro*. It is carcinogenic to rats and mice. It is similar to ethylene oxide structurally and chemically. It undergoes rapid acid hydrolysis (half-life of 1 min in 0.1 M HCl at 37°C). *In vivo*, the product of PO hydrolysis is 1,2-propylene glycol. Absorption occurs through the lung and gastrointestinal tract; because of its low molecular weight and reactivity, the dose may be radiomimetic throughout all organs.

DNA adduct studies have been carried out both *in vitro* and *in vivo* with PO. Because of the chiral nature of carbon 2 of PO, at least four different isomers can result during adduct formation. No known DNA adducts have been isolated from *in vivo* exposures, and the expected 7-(2-hydroxypropyl)-guanine adduct was not observed when mice were

injected with radiolabelled PO. Adducts with hemoglobin have been observed.

Propylene oxide is formed from propene gas by oxidative metabolism in male CBA mice. Thus alternative sources of PO exist and may confound estimates of environmental exposure.

1.3.6 4,4'-Methylenebis-2-Chloroaniline

4,4'-Methylenebis-2-chloroaniline (MOCA) is mutagenic to Salmonella; it is carcinogenic to rats, mice, and probably to dogs. Because of its structural similarity to aniline, it is a suspect bladder and liver carcinogen for humans. MOCA is absorbed through the skin or, upon inhalation, by the lungs. Rats excrete 50% of a skin dose within 72 h while humans excrete a larger fraction. Genetic differences in ability to detoxify by acetylation may play a role in the persistence of absorbed MOCA in humans. Some of the MOCA is excreted into urine unchanged while the major portion is excreted in the feces. Humans excrete a lower proportion of unchanged MOCA than do rats. Studies with labelled MOCA in rats indicate that retention of the label occurs in the liver and adipose tissue (white fat).

The metabolic pathway for detoxification appears to parallel detoxification of aromatic amines with acetylation and oxidation of the ring position next to the exocyclic amino group. Dogs excrete the sulfate ester, 5-hydroxy-3,3'-dichloro-4,4'-diaminodiphenylmethane-5-sulfate. Humans do not excrete the sulfate ester, but rather the mono- and diacetates (N-acetyl-MOCA and N,N'-diacetyl-MOCA).

Only one study was found dealing with DNA adduct formation by MOCA or its metabolites. The hydroxylated sulfate metabolite formed by dogs was found to bind to DNA *in vitro*. No identification of the formed adduct was made. Exposure to MOCA also causes DNA damage to hepatocytes from mice, rats, and hamsters.

1.3.7 Direct Blue 6, Direct Black 38, Direct Brown 95, and Benzidine

The benzidine dyes, Direct Blue 6 (DBU), Direct Black 38 (DBK), and Direct Brown 95 (DBR) are treated as a group since it is believed that reductive cleavage of the dyes yields free benzidine (BZ). Benzidine is a potent human carcinogen and can induce experimental cancer in animals. There is, however, some evidence that free benzidine diimine can undergo internal rearrangement to a benzidine-nitrenium intermediate that can then react with nucleophilic groups in DNA or protein. Thus it may only be necessary to hydrolyze the three toxic dyes to release the benzidine diimine group and, without external reduction, form the reactive nitrenium intermediate thought to be the ultimate carcinogenic form of BZ.

All three dyes are themselves carcinogenic in rats and mice. Benzidine is readily absorbed both upon inhalation and skin exposure. Workers in contact with DBK excrete BZ as well as 2,4-diaminoazobenzene. Human feces metabolize DBK anaerobically to BZ and three other compounds. Metabolism of BZ and BZ dyes proceeds by

acetylation and subsequent oxidation by either of two enzyme systems - microsomal P450-dependent oxidases or peroxidases.

Simple transformation of these carcinogenic dyes to BZ or its reactive intermediate may not account totally for their carcinogenicity. This is suggested by the fact that exposure to BZ dyes gives rise to a different set of DNA adducts than does exposure to BZ. The primary adduct isolated after exposure of animals to BZ, ABZ, DABZ, and the noncarcinogenic dye, Congo red, is N-(deoxyguanosin-8-yl)-N'-acetylbenzidine (N-dG-N'-ABZ). Injection of DBU resulted in N-dG-N'-ABZ and two additional adducts, N-(deoxyguanosin-8-yl)-N'-benzidine (N-dG-N'-BZ) plus the free base form and a larger adduct of undetermined structure that is believed to contain one of the amino-naphtholsulfonic acid groups of the original DBU. With time, the level of N-dG-N'-ABZ from BZ or Congo red exposure decreased in the liver, while the level of binding from DBU exposure increased over a period of seven days. There is still some uncertainty in the adduct formation mechanism from DBU; the two reports on DNA adduct isolation do not agree entirely on the identity and amounts of adducts formed.

1.3.8 Acrylonitrile

Acrylonitrile (AN) is mutagenic and is considered to be a probable human carcinogen based on limited evidence for carcinogenicity in humans and rats. Both the parent AN and its oxidized metabolite, 2-cyanoethylene oxide (CNEO), can react with DNA. Only one DNA adduct has been identified from *in vivo* studies: N^7-(2-oxoethyl)-guanine (7-OEG). A large number of DNA adduct-like products have been identified from *in vitro* studies and from isolation of urinary excretion products. These arise through loss of the cyanide group from the cyanohydrin formed from hydration of cyanoethylene oxide, hydrolysis of the nitrile group to carboxylic acids, rearrangement and decomposition of the several isomeric glutathione conjugates formed with CNEO, and by direct Michael addition of AN to the nucleophilic atoms of the individual bases.

Since 7-OEG is the only known adduct found from *in vivo* studies, and this adduct can be formed from vinyl chloride, chloroacetaldehyde, and other haloethylene compounds, use of this adduct for molecular epidemiological studies should be made with caution. In addition, this adduct has been shown not to cause miscoding in several *in vitro* assays, so that the linkage to a genotoxic event is weak.

1.3.9 Benzyl Chloride

Benzyl chloride (BC) appears to be weakly mutagenic to Salmonella and *E. coli* and to induce chromosomal aberrations. There is some suggestion of carcinogenicity in rats. Benzyl chloride is absorbed through the lungs and gastrointestinal tract. In rats, steady-state levels in blood can be achieved in less than 4 h of inhalation. The half-life of absorption is 0.53 h, and the half-life for elimination is 1.12 h. On intravenous injection, the peak plasma concentration is

reached within 15 min followed by a biphasic elimination with half-lives of 38.5 min and 33.2 h. Benzyl chloride is lethally toxic to female rats at 1/5 the exposure that is lethally toxic to male rats, and females maintain higher blood levels of radiolabelled BC than males. The mercapturic acid N-acetyl-S-benzylcysteine is the major urinary product from dogs, rats, and rabbits. Administration of radioactive BC by gastric incubation led to a tissue distribution in which the highest amounts were in the stomach and small intestine followed by kidney, liver, blood, lung, heart, pancreas, fat, and right femur muscle.

DNA adduct formation by BC has been studied primarily *in vitro*, but also *in vivo*. At least 5 DNA adducts are possible, resulting from benzylation at two N sites each for guanosine and adenosine and at O^6 for guanosine. Studies of the reaction of BC with individual nucleosides under several solvent conditions show that the rate and yield of the products are different depending on the ratio of organic solvent to aqueous solvent. It is not known if the same effect occurs in tissues.

An *in vivo* study showed that the second-order rate constant of formation of the N^7-(7-benzyl)-deoxy-guanosine (7-BG) in mouse spleen DNA is 3.3×10^{-4} $L \cdot (g\ DNA)^{-1} \cdot h^{-1}$. The corresponding rate for alkylation of hemoglobin in erythrocytes *in vitro* is 1.6×10^{-3} $L \cdot (g\ Hb)^{-1} \cdot h^{-1}$. Brain, testes, lung, liver, and pancreas contained radioactive 7-BG at 1-h postinjection. Accumulation continues over a 24-h period even though the half-life of BC in blood is about 1 min.

1.4 CONFOUNDING FACTORS

Additional factors need to be considered for molecular epidemiology studies. Concomitant exposures to other toxic, especially other genotoxic and/or carcinogenic, chemicals and to multiple sources of the same DNA adduct are two sets of potentially confounding situations. Individual exposure to several of the chemicals (ethylene oxide, styrene, vinyl chloride, epichlorohydrin, acrylonitrile, and benzyl chloride) is potentially confounded by concomitant exposure to other toxic chemicals. Some of the chemicals under consideration, including ethylene oxide, vinyl chloride, and acrylonitrile, do not produce unique DNA adducts; that is, the same adduct may be formed by exposure to other chemicals and thus may not necessarily be indicative of exposure to the substance of interest. These factors are presented in greater detail in Section 12.

2. ETHYLENE OXIDE

Ethylene oxide (EO) (CAS # 75-21-8) is widely used as a gas sterilant for animal bedding, medical instruments, and hospital equipment. It is considered to be a probable human carcinogen (IARC 1985). It has been shown to cause chromosomal damage in man (Thiess, Schwegler, and Stocker 1981) and animals resulting in increased frequency of sister chromatid exchanges (SCE) and chromosomal aberrations. Disease observations in experimental animal studies include carcinogenesis (cerebral gliomas, peritoneal mesotheliomas, and mononuclear cell leukemias), decreased fertility, increased fetal death, heritable chromosomal translocations, malformed fetuses, low sperm count, and low sperm motility. Human epidemiologic studies suggest that exposure at 20 ppm might be associated with excess spontaneous abortion and excess leukemia (Landrigan et al. 1984). The recommended time-weighted average threshold limit value (TLV/TWA) in the U.S. is 1 ppm ($\approx$2 mg/m^3) (ACGIH 1986e). In Sweden the TLV is 5 ppm (ACGIH 1986e). The OSHA permissible exposure limit in the U.S. is 50 ppm (ACGIH 1986e).

2.1 CHEMISTRY

Ethylene oxide is a gas at ambient temperature and boils near 12°C (Ehrenberg et al. 1974). The oxide is metastable with a half-life of 6 months in water at 4°C (Ehrenberg et al. 1974). However, the decomposition of the epoxide is acid catalyzed, so the observed lifetimes in biologically derived aqueous solutions may be shorter. The relative order of reactivity of EO with the target nucleophilic atom is sulfur > nitrogen > oxygen. The measured pseudo first-order rate constant for hydrolysis of EO at 37°C is 9.12×10^{-3} h^{-1} which results in a half-life of 76 h.

2.2 ABSORPTION

Gaseous ethylene oxide is absorbed into blood from the alveolar air spaces. Studies of 10 hospital workers (Brugnone et al. 1986) employed in the sterilizer unit showed a correlation between alveolar EO concentrations and blood concentrations ($r = 0.744$, $P < 0.001$), and both showed correlations to the environmental EO concentration ($r = 0.947$, $P < 0.001$; $r = 0.827$, $P < 0.001$). Alveolar retention of EO was 75 to 80% of the inhaled EO, while the blood/air partition coefficient was 90 ± 20 (S.D.) when measured by *in vitro* techniques. The mean blood/air ratio measured *in vivo* in exposed workers was smaller with values between 12 and 17. Measurements of blood and environmental EO in non-exposed workers showed that the blood/air ratio, on the average, was three. The environmental concentration of EO ranged from 0 to 25 mg/m^3 with the distribution skewed to the 1 to 10 mg/m^3 range.

Estimates of human dose based on environmental levels require a number of assumptions which include the values for human alveolar and pulmonary ventilation during exposure, the half-life of EO after uptake, and the actual length of time of exposure. Ehrenberg et al. (1974) have developed a concept for estimating tissue dose (dose to critical biological target) that bypasses these assumptions. The concept they have suggested is based on the idea that the toxic response is dependent on the reaction of the chemically reactive form of the toxicant. Thus measures of tissue reaction with the toxicant will be a direct measure of critical exposure. This subject is treated in more depth in Appendix A.

Dose-rate plays a significant role in mortality of exposed animals. An exposure dose of 3300 ppm·h in 4 h is an LD_{50} (dose rate of 825 ppm/hr), while many animal species show no adverse effects of daily exposures to dose rates of 350 ppm/h with a total dose of 45,000 ppm·h (Ehrenberg et al. 1974).

2.3 METABOLISM

2.3.1 Half-Life

In vivo, the initial half-life of EO in male CBA mice 6-8 weeks old was observed to be 9 min (detoxification and elimination) based on i.p. injection of EO (Ehrenberg et al. 1974). Segerback (1983) observed a 10 min half-life for EO in male CBA mice that were 8-12 weeks old. Osterman-Golkar et al. (1983) observed a half-life of 10 min after i.p. injection of EO into male Fischer 344 rats. The difference in half-life values between *in vitro* chemical hydrolysis and *in vivo* removal is probably due to chemical catalysis of hydrolysis and reaction with tissue nucleophiles.

2.3.2 Conversion of Ethene to EO

Ethylene oxide is formed from ethene *in vivo*; ethene is a significant component of cigarette smoke (Segerback 1983). Anderson et al. (1980) have demonstrated that ethene is converted to ethylene oxide in rats by an enzymatic process with a maximum conversion rate (V_{max}) estimated to be 0.24 mg/kg body weight per h. The equilibrium constant (K_m or one half the concentration needed to achieve the maximum rate) was 218 ppm in air. At low concentrations (0.25 to 11 ppm) Segerback (1983) found that the uptake of ethene by CBA mice had a half-life of 5 to 6 h. The amount of ethene converted to EO was estimated to be about 8% of the dose based on adduct formation. This agrees with the observation of Ehrenberg et al. (1977) of 7% conversion to EO. Segerback (1983) suggests that observed Vmax and Km values can be used to calculate the levels of ethylene oxide formed from ethene. Using the above values for Km and Vmax, Segerback (1983) estimates that the effective EO concentration, derived from the ethene exposure experiment of Ehrenberg et al. (1974), was 4 ppm. Ehrenberg et al. (1974)

calculate that 1 ppm·h of EO is equivalent to 2.5 μmol EO/kg body weight for the CBA mouse.

2.3.3 Urinary Metabolites of Ethylene Oxide

The major urinary metabolite derived from ethylene oxide exposure of adult Sprague-Dawley rats is 2-hydroxyethylmercapturic acid (N-acetyl-S-2-hydroxyethyl-L-cysteine) (Jones and Wells 1981). The measurement of the o-phthalaldehyde-amino acid complex of mercapturic acid in urine is suggested as a replacement for the lengthy and tedious measurement of 2-hydroxyethyl-amino acid derivatives from blood. The new procedure involves enzymatic deacetylation of aminoacid amides in urine, deproteinization with mercaptoethanol-methanol, then followed by derivatization, chromatography, and detection by fluorescence. The detection limit was 0.15 nmole in 10 μL of sample. With this technique an exposure of 6 ppm·h was detected as an excretion of 0.39 μmole of 2-hydroxyethylmercapturic acid in 24 h. The excretion was linear with dose up to 189 ppm for 6 h (the highest dose tested). On the average, 14% of the dose was recovered in the 24 h post exposure. Intravenous injection experiments gave about 35% of the dose in the 24 h urine collection. These results do not include losses by exhalation. They calculate that rats produce 0.045 μmole/ppm·h of the excreted mercapturic acid. Other chemicals are known to produce the same excretion product. They include vinyl chloride (Watanabe, McGowan and Gehring 1976), 1,2 dibromoethane (Van Bladeren et al. 1980), 2-bromoethanol (Jones and Wells 1981), and acrylonitrile (Van Bladeren et al. 1981).

2.4 DNA ADDUCT FORMATION

2.4.1 DNA Adduct Formation

The second-order rate constant is 0.9×10^{-4} L·(g DNA)$^{-1}$·h^{-1} for the reaction of EO with aqueous DNA *in vitro* at 37°C and 1.0×10^{-4} L·(g DNA)$^{-1}$·h^{-1} for reaction with DNA in intact mouse spleen cells (Ehrenberg et al. 1974). This indicates that packaging of DNA within the cell does not significantly affect the rate of alkylation. EO reacts with protein (interphase from phenol extraction step) about three- to fourfold faster than with DNA. The observed rates are (as k·L·g^{-1}·h^{-1}) 3.3×10^{-4} for liver proteins (*in vitro*), 2.9×10^{-4} for spleen proteins *in vitro*, and 4.3×10^{-4} for spleen protein from mouse spleen cell exposure (Ehrenberg et al. 1974).

The major DNA adduct formed (90% of the total) by reaction of DNA with EO is N^7-(2-hydroxyethyl)-guanine (N^7-[2-HOEt]-Gua) as shown in Table 2.1 (Ehrenberg et al. 1974). Osterman-Golkar et al.(1983) observed this adduct in DNA isolated from rat liver after different exposures (0.74 nmol/gm DNA at 20.4 μmol/kg bw, i.p.; 0.19 nmol/gm DNA for 2.8 μmol/kg bw); one-third of those amounts were found in the rat testes. The value 0.74 nmol/gm DNA corresponds to 2 adducts per 10^7

bases (about 1200 per cell genome). During the first 24 h post-exposure, 90 to 95% of the absorbed dose is collected in the urine. The N^7-(2-HOEt)-Gua adduct was 0.007% of the excreted radioactivity.

Table 2.1. DNA adducts formed from ethylene oxide

Reference	DNA source	Dose	Binding or half-life
	N^7-(2-hydroxyethyl)guanine adduct		
Osterman-Golkar et al. (1983)	Fischer 344 rats	0-100 ppm inh. 30 h/wk for 2 years	
	Fischer 344 rats	20.4 μmol/kg bw for 5 h	0.25-0.75[a] nmol/g
		2.8 μmol/kg bw for 5 h	0.07-0.19 nmol/g
Ehrenberg et al. 1974	Male CBA mice, 6-8 wks old; tissues; adduct detected in urine	1-35 ppm i.p <105 min	0.007%
Segerback 1983	Male CBA mice	0.01-0.044 mmol per/kg ethene or EO.	Spleen: 24 h Liver: 12 h Testis: 20 h
von Hofe, Kleihues, and Keefer (1986)	Male Fischer 344	0.07 mg/kg i.p. or 6.3 mg/kg	0.4μmol/mol guanine
	N^1-(2-hydroxyethyl)-adenosine adduct		
Windmueller and Kaplan 1961	*In vitro* with adenosine, ATP	excess EO	13.3 h for NAD alkylation
	Unidentified adducts		
Sega 1982	(C3H/101)F_1 germ cells	3 ppm·h	3 h plus 10% residue at 4 d

[a]This is equivalent to about 0.6 to 2 adducts per 10^7 bases.

DNA adduct repair or removal by cell loss is rapid, with variable rates of decay. This contributes to the different levels of adduct formation observed in different tissues. During a 2 to 48 h exposure (Segerback 1983), the levels of observed guanine adduct in mouse spleen ranged from a high of 0.88 nmol/g DNA at 2 h with a half-life of decay of 24 h (at a dose of 44 μmole/kg i.p.), to a maximum of 0.068 nmol/gm DNA at 5 h with a decay half-life of 32 h (at a dose of 8.7 μmol/kg i.p.). The two conditions were compared by extrapolation to the expected initial level of guanine adduct formation at time zero. The extrapolated value from the low-dose exposure was one-tenth the value for the high dose exposure, which was less than the expected ratio of 0.23 (8.7 μM/44 μM). For testes, a high of 0.092 nmol/gm DNA with a decay rate of 20 hr was observed with the 8.7 μmole/kg bw dose.

Ehrenberg et al. (1974) exposed CBA mice to doses equivalent to 0.03 to 2% of the LD_{50}. The half-life *in vitro* of the radioactivity remaining in DNA (90% of label was the major adduct) was about 340 h at 37°C. Segerback (1983) observed a 90-h half-life for the adduct in vitro using calf-thymus DNA. For comparison, the half-life of N^7-methylguanine and the N^7-ethylguanine adducts of DNA were described to be 110-155 h and about 95 h, respectively. *In vivo*, the half-life of the N^7-(2-hydroxyethyl)-guanine was observed to be 20 h. This short half-life is explained by enzymatic repair and DNA catabolism such as glycosylases that catalyze the removal of N^7-alkyl guanine residues from DNA.

While the N^7-(2-HOEt) Gua is 90% of the DNA adduct formed, O^6-(2-hydroxyethyl)guanine (O^6-[2-HOEt]-Gua) and adenines alkylated at the N^1, N^3 and N^7 positions are 200, and 20 to 25 times (respectively) less abundant (quoted from Segerback [1985] in Farmer et al. 1986) (see Table 2.1).

The evidence that EO reacts with intrachain phosphate groups to form a phosphotriester comes from Walles and Ehrenberg (1968). On comparison of the reversibility of heat denaturation of EO DNA derivatives and 2-methoxylated DNA, the EO derivative was less stable. This result suggested increased strand scission. As an explanation of how the increased strand scission could occur, they suggest that an intermediate phosphotriester is formed with the 2-hydroxyl group of ethylene glycol adjacent to the phosphodiester link. This is essentially the same alkali or heat labile vic-diol intermediate triester structure found during RNA chain breakage. They propose that this alkali-labile intermediate is the site of chain cleavage on heat denaturation.

2.4.2 Observed Binding of EO to Hb and Other Proteins

Ethylene oxide adducts to protein are found in many tissues after *in vivo* exposure (Ehrenberg 1974). Male CBA mice exposed to 1.44 ppm·h, integrated dose, of EO formed adducts in lung, liver, kidney, brain, spleen, and testis (Table 2.2). The amounts of adduct ranged from 0.45 nmol/g lung protein to 0.93 nmol/g liver protein. Radioactivity was found in both the phenol-soluble protein and insoluble material at the interphase after phenol extraction.

Table 2.2. Tissue levels of protein adducts in CBA male mice[a]

Animal/Tissue	Adduct level nmol hydroxyethyl/g	
	Phenol	Interphase
Lung	0.45	0.71
Liver	0.70	0.48
Kidney	0.93	0.55
Brain	0.54	-
Spleen	0.63	0.47
Testis	0.63	0.27

[a]Ehrenberg et al. 1974; integrated dose of 1.44 ppm·h.

Sega (1982) measured DNA adducts formed by exposure to 3 ppm·h of EO. In the developing germ cells of the mouse, the last scheduled DNA synthesis takes place during a 14-h period in preleptotene spermatocytes. After DNA synthesis is complete, these spermatocytes continue to develop until leaving the testis. Chemically-induced unscheduled DNA synthesis (UDS) can be detected, after the preleptolene stage, by injection of [^{3}H]-thymidine after chemical exposure. The sperm can be collected at any subsequent stage, including ejaculation, and the radioactivity can be used as a measure of UDS. This UDS response is expected to remove some or all of the DNA adducts formed during the exposure. Sega (1982) presented data showing rapid loss of radioactive EO-DNA derivatives. He observed that about 10% of the label remains at 4 days post exposure, and we have estimated from his data that the half-life of removal in the early post-exposure interval is about 3 h.

Segerback (1983) observed that EO reacts with CBA mouse blood cells *in vitro* to form N^{τ}-(2-hydroxyethyl)-histidine at a mean rate of 0.17 $L\cdot(g\text{-}Hb)^{-1}\cdot h^{-1}$ at 37°C over a 40-min period. The relative rates of alkylation of the N^{π}-His-, Cys-S- and Val-NH_2 compared to the N^{τ}-His adduct showed relative rates of synthesis of 1.5-, 2.9-, and 1.5-fold higher values, respectively, than the His-N^{τ} adduct. An examination of the several experiments providing these results suggests that there is considerable variation from these average values depending on the exposure dose.

A number of studies support the idea that Hb adduct formation can be predictive of exposure. Ehrenberg et al. (1977) studied the conversion of ^{14}C-ethene to cysteinyl- and histidinyl adducts of Hb. The extent of alkylation was consistent with the expected levels of metabolic conversion of ethene to EO. Hemoglobin adducts to histidine and cysteine, and adducts to DNA guanine residues, are formed from ethene exposure in

essentially the same proportion as when EO is injected (Segerback 1983). The proportion of adducts formed by ethene in blood, liver, spleen, and testis were also similar to the proportions found when EO was injected. The value of that ratio ranged from 2.6 to 10. The theoretical ratio based on reaction rates of EO with Hb and DNA *in vitro* is 5.6. The variability in predicting the adduct ratio is thus approximately ± 100%, and the uncertainty in predicting the level of DNA adduct formation from the level of Hb alkylation can be as high as a factor of 4 (uncertainty is the ratio of the extremes for the ratio of DNA-adduct formation to HB-adduct formation = 10/2.6). A second example comes from the work of Osterman-Golkar et al. (1983). They demonstrated an essentially linear dose-dependent production of N^3-(2-hydroxyethyl)histidine in rat hemoglobin on continuous exposure to EO at 10, 33, and 100 ppm for a period of 2 years.

Calleman (1982) described *in vivo* dosimetry using Hb adduct formation as a measure of *in vivo* dose compared to the external dose rate in rats chronically exposed to either EO or ethylene. The curve shape is nonlinear with slope decreasing at increased dose-rate. The absorbed dose from ethene showed saturation kinetics as expected if the dose exceeds V_m for conversion of ethene to EO. The plotted data from EO exposure study approached saturation but did not achieve a saturation type curve structure.

2.4.3 Human Dosimetry

The observed level of N-hydroxyethylvaline in human hemoglobin isolated from cigarette smokers compares favorably with the expected amount based both on inhalation of ethene as the primary source of EO and based on rodent dosimetry (Tornqvist et al. 1986) (see Table 2.3). The mainstream smoke contains about 0.24 mg ethene per cigarette, and the smokers are assumed to smoke 30 cigarettes per day. The study group smoked more than 20 cigarettes per day. Previous studies have shown that exposure of animals to 1 mg/kg ethene gives rise to a tissue dose (*D*, see Eq. A.1 in Appendix A) of 0.7×10^{-6} M·h EO. The rate constant for reaction of EO with the terminal valine of Hb is 0.5×10^{-4} $L \cdot g\ Hb^{-1} \cdot h^{-1}$. Appendix A contains a more detailed discussion of the estimation of dose from the degree of DNA or Hb alkylation and the potential use of adduct formation in exposure monitoring.

Other sources of N^2-hydroxyethyl groups in cigarettes may include the smallamount of diethanolnitrosamine present in cigarette smoke or the spurious ethene production from oxidized lipids. They may also arise from microbial metabolism or from methionine metabolism (Tornqvist 1986).

Adduct formation in rats exposed to nitrosomethylethylamine (NMEA) was shown to give rise to N^7-(2-HOEt)Gua at a level of about 2% of the total adducts (von Hofe, Kleihues, and Keefer 1986). The effort to demonstrate the 2-hydroxyethyl derivative was based on an earlier observation that nitrosodiethylamine led to formation of hydroxyethylprotoporphyrin IX (White, Smith, and Farmer 1983). These

Table 2.3. Hemoglobin adducts

Animal/Adduct	Level nmol/g Hb	Dose	Formation 10^4 L·(g Hb)$^{-1}$·h^{-1}	Decay
CBA mouse/ Histidine-N^{τ}[a]	0.032-0.32	8.7-44[b]	0.17	-0.17
Fischer 344 male rats/Histidine[c]	1.3-2.8	0		
Fischer 344 male rats/Histidine-N^{τ}[c]	14-82	10-100[d]	0.27 ± 0.05	
Human nonsmokers[e]	0.058 ± 0.025	"0"		
Human smokers[e]	0.389 ± 0.138	>20 cig/d		
Theory from ethene in cig[e]	0.24			

[a]Segerback 1983.
[b]μmol/kg.
[c]Osterman-Golkar et al. 1983.
[d]ppm.
[e]Tornqvist et al. 1986.

studies suggest that metabolic β-hydroxylation of the diethylnitrosamine or some later product could be occurring, and the pathway leading to the N^7-(2-HOEt)Gua may be a minor one. If a similar metabolic pathway exists in humans, then exposure to certain nitrosamines may potentially confound a study of EO exposure and adduct formation.

2.5 DNA DAMAGE STUDIES

Pero et al. (1981) studied the effect of human EO exposure on the ability of lymphocytes (including monocytes) to exhibit normal DNA repair and chromosomal aberrations. Several assays were applied to two exposure groups in a factory manufacturing disposable medical products. The workers fell into two dose ranges: packers were exposed to 0.5 to 1 ppm over an average of 4 years; lab technicians and sterilizers were exposed to 5 to 10 ppm over an average of 1.6 years. A control group was made up from workers assembling the products.

Total chromatid gaps plus breaks were significantly elevated, while the ability to express UDS after stimulation of lymphocytes *in vitro* by N-acetoxy-2-acetylaminofluorene (NAAAF) was negatively correlated to exposure. This was verified by *in vitro* incubation of nonexposed lymphocytes with EO prior to testing for UDS. Sensitization of lymphocytes to the negative induction of UDS was observed at 0.1 μM to >2mM concentrations. Cytotoxicity was also observed to increase to 40% compared to 5% for controls when sensitized cells were exposed to NAAAF. The authors conclude that cells surviving EO plus NAAAF were inhibited in their DNA repair capacity as judged by UDS.

The effective concentration of EO in blood vs isolated leucocytes or lymphocytes was different. While isolated cells showed a maximum in UDS activity between 1 and 2 μM, whole blood required 5 μM EO. The results are also complicated by the differing levels of lethal cytotoxicity associated with EO exposure.

An analysis of the environmental levels of EO compared to localized concentrations of EO in blood suggested that cumulative effects must occur in the workplace in order to have the UDS effect expressed. This conclusion is based on estimating the effective concentration of EO for threshold limit value exposures (TLV) based on the studies of Ehrenberg et al. (1974). Pero et al. (1981) calculate that the exposure limits (50 ppm for USA and 10 ppm for Sweden) correspond to 5 μM and 1 μM EO daily tissue doses. From their experimental observations, they show that the *in vitro* UDS effect occurs at 0.3 to 10 mM. This hundred fold higher concentration required for *in vitro* UDS inhibition is the basis for suggesting that cumulative toxic effects may occur. The effect of EO on UDS is reversible; removal of the employee from EO for 9 months restored the normal UDS response.

Widegren et al. (in Pero et al. 1981) state that they have found a mathematical relationship between EO exposure and *in vitro* cytotoxicity:

$$\text{cytotoxicity} = \text{constant} \cdot (1/([EO] + 1)) + b \qquad (2.1)$$

The bimodal shape of the cytotoxicity curve may help explain the peculiar pharmacokinetics of UDS inhibition.

3. STYRENE

Styrene (ethylenebenzene, CAS # 100-42-5) is used in the manufacture of plastics, rubber, and resins. The permissible occupational exposure limit is 100 ppm (NIOSH 1985). Studies with mice and rats indicate that excess lung tumors, leukemia, and lymphosarcomas are induced by exposure to styrene. Exposure of rats to styrene oxide by gavage results in a potent direct carcinogenic response (Vainio et al. 1981; Maltoni, Failla, and Kassapidis 1979). Limited epidemiological evidence exists for reproductive effects due to human exposure to styrene, but considerable evidence exists for cellular mutagenicity and chromosomal damage (Vainio et al. 1981).

3.1 CHEMISTRY

Styrene is a colorless liquid boiling at 145°C and is slightly soluble in water. It is believed that its toxicity is due to the oxidation of styrene to an epoxide. Two types of reactions are possible: ring oxidation to an arene oxide or oxidation of the vinyl side chain to an alkyl epoxide. Both epoxides can react with electrophiles to yield a variety of products. The metabolic products have been the focus of major study.

3.2 ABSORPTION

The rates of several variables involved in styrene absorbtion have been measured (Table 3.1). A number of studies show that inhaled styrene is retained at levels of 60 to 70% of the dose over an 8 h period (Vainio et al. 1981, Wieczorek and Piotrowski 1985).

Most of the absorbed styrene is cleared by the metabolic route. Under conditions of human-volunteer exposure to 80 ppm in air for 6 h, clearance of styrene follows a two-compartment linear pharmacokinetic model (Ramsey et al. 1980). Individual differences in mean lung ventilation values can range from 0.4 to 0.7 m^3/h (Wieczorek and Piotrowski 1985). The individual absorbed dose can be estimated from Eq. (3.1):

$$D = C \cdot T \cdot R \cdot V \tag{3.1}$$

where D is the absorbed dose; C is the average styrene concentration in air as mg/m^3; T is the exposure time in h; R is the average fractional retention of styrene in the lungs; and V is the average lung ventilation in m^3/h. These data have been correlated with levels of urinary excretion of styrene metabolites. Air levels of 20 mg/m^3 will result in measurable levels of urinary metabolites, and a level of 100 mg/m^3 corresponds to excretion of mandelic acid at a rate of 15 mg/h.

Percutaneous absorption of styrene was measured in volunteers by dipping the hand in liquid styrene for 10 to 30 min followed by periodic measurement of metabolites in body fluids (Berode, Droz, and Guillemin

Table 3.1. Constants for absorption of styrene by humans[a]

Test system	Value	Dose
Liquid styrene on skin → urine metabolites	1 ± 0.5 $\mu g/(cm^2 \cdot min)$	
Liquid styrene on skin → styrene in tissue	150-250 $\mu g/(cm^2 \cdot min)$	
Inhalation → subcutaneous adipose tissue	50 $\mu mol/kg$ bw	150 $mg/m^3 \cdot h$ for 2 h
Inhalation → arterial blood	20 μM	150 $mg/m^3 \cdot h$
Ratio of totals [inhaled-exhaled/ inhaled]	0.7	below 200 mg/m^3
Ratio: blood/alveolar air at 37°C	61	
Ratio: blood/inspired air	7.5	
Acute changes		
Blood clearance	1.7 ± 0.3 L/min	150 $mg/m^3 \cdot h$
Elimination half-life	41 ± 7 min	150 $mg/m^3 \cdot h$
Volume distribution	99 ± 13 L	
Half-life: styrene glycol elimination	72 ± 13 min	
Half-life: styrene in subcutaneous adipose tissue	72 h	

[a]Taken from Wieczorek 1985, Wigaeus et al. 1983; Berode, Droz, and Guillemin 1985; and Lof, Gullstrand, and Nordqvist 1983.

1985). The initial rate absorption measured in these experiments was 1 ± 0.5 $\mu g/(cm^2 \cdot min)$, and it decreased with time of exposure. The amount absorbed was estimated from the urinary production of mandelic and phenylglyoxylic acid. These values are considerably smaller than those reported earlier by Dutkiewicz and Tyras (1967, 1968), who measured the disappearance of styrene from aqueous solutions when hands were immersed in the solution for periods of one hour at room temperature. The

differences between the two studies may be related to the storage of styrene in subcutaneous adipose tissues without metabolism to the expected excretion products.

Wigaeus et al. (1983) observed that styrene in subcutaneous adipose tissue and blood is not in equilibrium after 2 h of exposure. The concentration of styrene reached 50 μmol/kg within 0 to 90 min after exposure. The half-life of styrene in subcutaneous adipose tissue is 2.2 to 5.2 days. These observations support the absorbtion rate values of 9 to 15 mg/(cm^2·h) (150 to 250 μg/[cm^2·min]) reported by Dutkiewicz and Tyras (1968). These studies also indicate that the daily level of excretion products is not necessarily the total absorbed dose.

Wigaeus et al. (1983) measured the *in vivo* steady state partition coefficient of styrene between human arterial blood and inspired air (N_{eff}) using the following equation:

$$N_{eff} = N \,/\, [1 + E_t \cdot (Q'_t \,/\, V'_A) \cdot N] \qquad (3.2)$$

where N is the thermodynamic blood:air partition coefficient at 37°C ($N = 61$); ($Q'_t \,/\, V'_A$) is the ratio of cardiac output to alveolar ventilation rate ($Q'_t \,/\, V'_A$ = 9 L/min / 17 L/min); and E_t is the systemic extraction (fraction in tissue based on the concentration differences between arterial blood and venous blood divided by the concentration in arterial blood).

Styrene oxide is rapidly cleared from blood after intraperitoneal injection of 200 mg/kg into male CD_2F1 mice. The observed rate constant of absorption was $K_a = 0.288\ min^{-1}$; the rate constant of elimination $K_{el} = 0.202\ min^{-1}$; the half-life = 3.4 m; the concentration, extrapolated to time zero, C_o = 224 μg/mL; and the apparent volume distribution V_d = 2.99 L/kg (Bidoli, Airoli, and Pantarotto 1980). The acute excretion half-lives of mandelic acid and phenylglyoxylic acid are, respectively, 3.6 ± 0.4 h and 8.8 ± 1.3 h during a 0 to 20-h period of exposure.

3.3 METABOLISM

The liver is the primary route of metabolism with excretion of the metabolites and conjugates occurring primarily through the kidney (Korn et al. 1984). Ramsey et al. (1980) studied the pharmacokinetics of styrene and suggested that clearance from the body follows a two compartment linear model when human volunteers are exposed to 80 ppm for 6 h. Whole blood can convert styrene to styrene oxide, and exposed lymphocytes show both SCE and chromosomal aberrations (Vainio et al. 1981).

Intraperitoneal injection of ^{14}C-styrene (Lof, Gullstrand, and Nordqvist 1983) into NMRI mice results in the distribution of styrene into liver, kidney, lung, subcutaneous adipose tissue, brain, pancreas, spleen, and testis within 2 h. In the dose range of 1.1 to 4.9 mmol/kg body weight, the highest initial concentrations of unchanged styrene were found in adipose tissue, pancreas, liver, and brain. The levels of

styrene glycol were highest in kidney, lung, pancreas, and liver, with much lower levels in subcutaneous adipose tissue. Styrene glycol was found in conjugated form in liver and kidney, while other polar metabolites were observed in lung and plasma as well as in liver and kidney. The accumulation of unmetabolized styrene increased in all tissues with increasing dose. The slope of the accumulation in subcutaneous adipose tissue was four-to tenfold higher than the other tissues. This may reflect the apparently slower rates of metabolism of styrene in this tissue. The half life for styrene in adipose tissue is estimated at 6 h in rat (Lof, Gullstrand, and Nordqvist 1983) and about 72 h in man (ibid.).

Lof et al. (1986) measured styrene metabolites in the blood of 10 men occupationally exposed to styrene in two glass-fiber reinforced plastics factories. The mean external level of exposure was 99 mg/m^3. Blood was removed from a brachial vein at 7 AM, 9 AM, and 12 noon in plant 1, and samples were taken at 5 PM and 6 PM at plant 2 (the end of the work day). Blood was extracted with hexane (for styrene oxide), then ethyl acetate (for styrene glycol), and the residual aqueous solution was tested for conjugates by treatment with β-glucuronidase. Each was assayed by gas chromatography/electron capture after conversion to styrene glycol and derivatization with pentafluorobenzoyl chloride. The limits of detection of styrene oxide and conjugated styrene glycol were 20 nM, and the limit of detection of free styrene glycol was 80 nM. Styrene oxide was observed at the limits of detection (20 nM), while styrene and styrene glycol were observed to increase with increasing dose.

Two metabolic pathways have been suggested: the major pathway with considerable documentation is through the styrene-7,8-oxide with subsequent hydrolysis and oxidation of the side chain; the minor pathway is through arene oxide formation of styrene 3,4-oxide (Vainio et al. 1981).

Styrene is metabolically oxidized by P450-dependent monoxygenases in liver to a 40/60 mixture, respectively, of D and L styrene-7,8-oxide (Korn et al. 1984). Both enantiomers are either hydrolyzed by epoxide hydrase to the corresponding styrene glycol or react with glutathione to form mercapturic acids. Styrene glycol is subsequently either oxidized to the carboxylic acid, D and L-mandelic acid, or conjugated to glucuronic acid. The chiral 7-OH group of mandelic acid is then oxidized to a ketone to form phenylglyoxylic acid. The main human metabolites found in urine are D and L-mandelic acid (55%) and phenylglyoxylic acid (35%) (Vainio et al. 1981).

Rabbit pulmonary microsomes oxidize 98% of supplied styrene by the combination of three isozymes of the P450-dependent monooxygenases (Harris et al. 1986). The rate and stereoselectivity of the reconstituted pulmonary microsomal oxidase system agreed well with the experimentally determined values and with the sum of properties of the individual isozymes.

The minor metabolic pathway of styrene-3,4-oxide formation is supported by the isolation of 4-vinylphenol from human and rat urine (Vainio et al. 1981, Bakke and Scheline 1970). The 4-vinylphenol, like

phenol formation during metabolism of polynuclear aromatic hydrocarbons, is formed by spontaneous rearrangement of styrene-3,4-oxide. The relative amount of 4-vinylphenol formation has been found to be 0.03% of the amount of urinary mandelic acid (Vainio et al. 1981).

The combined action of hemoglobin (via methemoglobin) or myoglobin (via metmyoglobin) with oxygen and H_2O_2 on styrene results in the formation of partially racemic styrene oxide (Ortiz de Montellano and Catalano 1985). Both oxygen and H_2O_2 independently oxidize styrene. The extent of this reaction in tissues is not described, but they conclude that the mechanism of oxidation is different than in P450-dependent monooxygenase catalyzed oxidations. Two products are formed: styrene oxide and benzaldehyde. The benzaldehyde is not derived from a secondary oxidation of styrene oxide but is formed in an essentially constant fraction of the total conversion of styrene to styrene oxide.

Styrene-glutathione adducts are formed on incubation of prostaglandin H synthase with styrene and H_2O_2 or arachidonic acid (Stock, Bend, and Eling 1986). The term adduct is used to indicate that the mechanism of formation does not involve the intermediate formation of styrene oxide. The reactive intermediate is believed to be a thiyl radical which adds to the C-8 position, leaving a radical at the C-7 position, followed by oxygen addition to the C-7 radical. The organic peroxy radical is then cleaved to a hydroxyl group. The only product formed is the C-7 hydroxy, C-8 glutathionyl adduct. These compounds were named as glutathione derivatives as (2R)- and (2S)-S-(2-phenyl-2-hydroxyethyl)glutathione. The corresponding (1R)- and (1S)-S-(1-phenyl-2-hydroxyethyl)glutathiones were not formed.

Seutter-Berlauge et al. (1978) found three S-containing styrene metabolites in rat urine: N-acetyl-S-(1-phenyl-2-HOEt)cysteine; N-acetyl-S-(2-phenyl-2-HOEt)cysteine; and N-acetyl-S-(1-phenylacetyl)cysteine. These three derivatives account for about 11% of the administered dose.

Korn et al. (1985) measured the enantiomer distribution of styrene metabolites in the urine of styrene-exposed (about 100 ppm, 9 h shift) and nonexposed populations. They observed the concentration of hydroxylated styrene metabolites to range from about 2 mg/L in nonexposed males to about 60 mg/mL in occupationally-exposed females after enzymatic release of the styrene derivatives. Taking the uncertainty of the data into account, there were no significant sex differences. The level of 1-DL-phenylethanol was below the detection limit in nonexposed males but was detected in some of the exposed individuals (0.8 ± 0.6 mg/L in 8 of 11 males and 0.2 ± 0.2 in 2 of 6 females). 2-Phenylethanol was observed in both exposed and nonexposed groups with a baseline level of 0.2 ± 0.2 mg/L in the nonexposed group, values of 1.4 ± 0.3 mg/L in the exposed males, and 0.9 ± 0.8 mg/L in the exposed females. The main excretion products were the L- and D-phenylethyeneglycols which were excreted in the ratio of 2.9 for males (L/D ratio) and excreted in a ratio of 3.2 for females, while nonexposed males gave a L/D ratio of 1.5. The ratio values were significantly different, and the authors suggest that studies should be done to establish the correlation between L/D ratio and the extent of exposure.

Styrene metabolism also produces significant quantities of carbon dioxide in the first day of exposure (Danishefsky and Willhite 1974). This can confound the results of determining radioactive DNA adduct formation since 8[^{14}C]-styrene can give rise to the 1-carbon fragment used for *de novo* purine and pyrimidine synthesis (Legraverend et al. 1984).

3.4 DNA ADDUCTS

The DNA adducts formed from radioactive styrene injected (i.p. in DMSO/corn oil) into mice (white Naval Medical Research Institute [NMRI]) were observed to be primarily the N^7-alkyl guanine derivative(s) (see Table 3.2) (Byfalt Nordqvist et al. 1985). The detection was made by co-chromatography with a synthetic mixture of isomers. The coincidence of radioactivity and the markers (α,β N^7-alkylguanines) was good; however, the amounts of radioactivity were quite low, i.e. 9 cpm above a background of 23 cpm (counted to 15% accuracy). These low counts preclude identification of minor adducts.

The time course of adduct formation was measured over a dose range of 1.1 to 4.9 mg/kg bw. The time of sacrifice was from 0.5 to 5 h post-exposure in the several experiments (see Table 3.2). The products identified from the reaction of radioactive styrene oxide with DNA *in vitro* were primarily N^7-(7[8]-hydroxyethylphenyl)guanine (83%) and N^2-(7[8]-hydroxyethylphenyl)guanine (Savela and Hemminki 1986, Savela, Hesso, and Hemminki 1986) (Table 3.3). The relative rates of alkylation with styrene oxide decreased in the following order: deoxyguanosine > single-stranded DNA > double-stranded DNA (Hemminki 1979) when measured using a fluorescence assay which detects emissions at 400 nm after excitation over the range of 220 nm to 360 nm. The reaction of styrene oxide with DNA in 30% ethanol was essentially complete in about 3 h at 32°C.

In vitro reaction of styrene oxide with deoxynucleosides resulted in nine different products with the following order of yields when the reaction was carried out at pH 7.4 with an excess of styrene oxide (87 mM): dG > dC > dA > Thd (Savela and Hemminki 1986; Savela, Hesso, and Hemminki 1986). Each deoxynucleoside gave rise to one or more derivatives (Table 3.4). There are four possible isomers that can be formed from each N- or O- site on the bases since the epoxide can react at the C-7 (α) or C-8 (β) position, and the C-7 position is chiral and can form two diastereoisomers. The C-7 position has a benzylic character and undergoes an S_N1 reaction mechanism that results in formation of both possible isomers. The C-8 carbon acts primarily through an S_N2 mechanism. Adduct formation at the guanine N^2- position occurs only with the C-7 (α) and is explained because it is such a weak nucleophile. The guanine O^6-position reacts with both C-7 (α) and C-8 (β) and favors reaction with C-7. For guanine, the ratio of substitution at C-7 over C-8 is 4.4. The explanation is given that the O^6- nucleophilicity is intermediate between that of guanine N^7 and N^2 (Hemminki et al. 1986).

Table 3.2. ***In vivo*** **adducts derived from styrene[a] and styrene oxide**

Dose mmole/kg bw	Time of sacrifice h	Organ	Adducts[b] Protein: Plasma[d]	Protein: Hemoglobin[e]	DNA[c]
		Styrene			
0.12	5	Blood	33		
0.71	5	"	160		
1.2	5	"	370		
2.4	5	"	370		
3.3	5	"	560		
1.1	2	"	200	3	17 ± 5
2.3	2	"	260	10	25 ± 9
3.3	2	"	290	40	
4.9	2	"	430	60	31 ± 6
		Styrene oxide			
0.037	2	Blood	9.8	0.1	
0.063	2	"	22	0.1	
0.092	2	"	25	0.4	
0.32	2	"	180	3.4	
1.1	2	"	750	13	8 ±2
0.36[f]	2	Brain			5 ±
0.36[f]	2	Lung			3 ± 1
0.36[f]	2	Spleen			0.6 ± 0.1
0.36[f]	2	Testis			0.3 ± 0.3

[a]Injected i.p. in DMSO/corn oil. The metabolism of styrene was incomplete at time of sacrifice, so the observed values may not represent maximum values. From Byfalt Nordqvist et al. (1985).

[b]All values as nmole per g. This corresponds to adducts per 3 x 10^6 DNA bases. The radioactive assay is insensitive with a limit of detection of 3 adducts per 10^7 base pairs when 2 to 8 mg of DNA are analyzed.

[c]This is the N^7-alkylguanine adduct(s) measured using 2 to 8 mg samples of DNA.

[d]Plasma protein values were determined from total acid insoluble plasma protein.

[e]Hemoglobin values were obtained from the globin portion of Hb isolated from washed red cells.

[f]Pooled DNA from exposures to 0.063 - 1.1 with an average of 0.36 mmol/kg bw. The samples were counted for 300 minutes.

Table 3.3. DNA adducts formed from styrene oxide[a]

DNA source	Adduct Formed	Yield
Salmon testes	N^7-(7-hydroxy-8-phenylethyl)guanine(α)	34%
	N^7-(8-hydroxy-7-phenylethyl)guanine(β)	49%
Salmon testes	N^2-[(α,β)-hydroxyethylphenyl]guanine	6%
	Unidentified	15%

[a]From Savela and Hemminki (1986) and Savela, Hesso, and Hemminki (1986).

Table 3.4. Deoxynucleoside reaction products with styrene oxide[a]

Deoxynucleoside	Product Formed[b]
Thymidine	Two 3-alkyl derivatives(100%) O^4-alkylthymidine[c]
Deoxycytidine	3-Alkyldeoxycytidine (55%) 4-Alkyldeoxycytidine (trace) O^2-alkyldeoxycytidine (45%)
Deoxyadenosine	1,(1(2)Hydroxyethyl-2(1)phenylethyl)-deoxyadenosines (40%) ± N^6-(2-hydroxy-1-phenylethyl)-deoxyadenosines (60%) 3-Alkyladenine[c]
Deoxyguanosine	N^7-alkyldeoxyguanosines (31%) N^7-alkylguanine (35%) N^2-alkyldeoxyguanosine (28%) O^6-alkyldeoxyguanosine (6%)

[a]Reaction of styrene oxide with deoxynucleosides, from Savela and Hemminki (1986) and Savela, Hesso, and Hemminki (1986).

[b]The number in parentheses is the percent of product for that nucleoside.

[c]This product was isolated after reaction in glacial acetic acid.

Reaction of deoxyadenosine and thymidine with styrene oxide in glacial acetic acid resulted in the formation of a pair of adenine alkylation products and a single O^4-alkylthymidine peak not found in the reaction at pH 7.4 (Hemminki and Suni 1984).

Reaction of guanosine at pH 7.4 gave rise to guanosine derivatives with the same substitutions as deoxyguanosine but with small differences in the quantitative distribution. No alkylguanine products were identified. The products of reaction of styrene oxide and 9-methylguanine showed the formation of N^7-, N^2-, and O^6- adducts (Hemminki and Hesso 1984).

3.5 PROTEIN ADDUCTS

The protein adducts formed from styrene injected i.p. into mice (NMRI, white, 25 g) included radiolabelled plasma proteins and hemoglobin (Byfalt Nordqvist et al. 1985) (see Table 3.2). The level of adduct formation at the N-terminal valine of Hb was measured by a modified Edman procedure that uses pentafluorophenylisothiocyanate to react with the terminal imine adduct. An internal standard was used to identify the position of the released thiohydantoin for radioactive counting. The maximum formation of plasma protein adduct from styrene oxide occurred within one hour and decayed with an essentially linear decay curve with a half-time of about 6 to 7 h. The binding derived from the injection of styrene reaches a maximum between 5 and 25 h (no points measured in between). At 25 h the binding level has fallen to the value observed at 1 h.

The nonlinear relationship observed by Byfalt Nordquist et al. (1985) between dose and adduct formation is not explained with additional data. The role of pharmacokinetic parameters is discussed, and limiting variables may include incomplete metabolism or saturation of the metabolism of styrene and altered rates of elimination at higher dosages.

3.6 DNA DAMAGE

Single strand breaks (SSB) were observed in liver, lung, kidney, brain, and testes of male NMRI mice (25 g) for both styrene and styrene oxide (Solveig Walles and Orsen 1983). These were observed at high doses (2 to 10 mmole/kg bw). Both styrene and styrene oxide gave peak values of SSB at about 4 h post exposure with final values taken at 24 h. Lof et al. (1983) and Weigaus et al. (1983) observed uptake of styrene and styrene metabolites into fatty tissue, and the stored styrene was not in equilibrium with blood at 2 h post-exposure. At the high doses used in the study for SSB formation, a continuous mobilization of the styrene or styrene oxide from the fatty tissue could modulate or induce an apparent persistence in SSB.

4. VINYL CHLORIDE

Vinyl chloride monomer (VC) (CAS # 75-01-04) is a colorless gas which boils at -14°C and is used primarily to make polymeric plastics. Polymerization takes place in the presence of light or other catalysts. It is soluble in polar organic solvents and benzene but is only slightly soluble in water.

The acute toxic effects in humans from high exposure include dizziness and loss of consciousness, and long term human effects include acro-osteolysis and angiosarcoma of the liver (reviewed by Purchase, Stafford, and Paddle 1987). Other known agents that have been associated with angiosarcoma are thorium oxide, arsenic, and possibly anabolic steroids. The lowest doses of vinyl chloride that give rise to liver angiosarcoma are 50 mg/kg or an airborne concentration of 200 ppm. The carcinogenic activity and dose response relationships for rats are well documented (Maltoni et al. 1981). The main organs involved in carcinogenesis in man are liver, lung, and brain. The ACGIH recommended TLV-TWA is 5 ppm (≈10 mg/m^3) (ACGIH 1986h); the permissible OSHA exposure limit is 1 ppm per 8 hr and 5 ppm (15-min ceiling) (NIOSH 1985).

4.1 METABOLISM

The primary metabolic pathway observed is the epoxidation of VC to chloroethylene oxide (CEO), which then either reacts with nucleophiles or rearranges to chloroacetaldehyde (CAA). The epoxidation is catalyzed by microsomal mixed function oxidases (Laib 1982). The maximum conversion rate for Wistar rats is 110 $\mu mol \cdot h^{-1} \cdot kg^{-1}$ (Bolt 1984). Because of the spontaneous rearrangement of CEO to CAA, 2,2'-dichlorodiethylether (DDE) was studied as a surrogate for formation of CAA with subsequent formation of macromolecular adducts. The metabolic products of DDE are CAA and 2-chloroethanol, which are subsequently converted to chloroacetic acid and excreted, in urine, as thiodiglychollic acid after intermediate glutathione conjugation. Conjugation of CEO with glutathione leads to urinary excretion of N-acetyl-(2-hydroxyethyl)cysteine (Gwinner et al. 1983).

Laib and Bolt (1977) observed that radioactivity from 1,2 ^{14}C-VC was incorporated into the natural nucleosides of rat liver RNA over an 86 h period post exposure, which indicated conversion of the radioactivity to normal precursors of purine and pyrimidine synthesis. More recent studies using radioactive tracers also showed some incorporation of [^{14}C]VC into deoxyguanosine, which suggests some metabolic breakdown of VC to 1-carbon fragments that can be incorporated into DNA (Laib, Gwinner, and Bolt, 1981). These results suggest caution in interpretation of incorporation of radioactivity into nucleic acids without identification of the specific radioactive compound(s).

Gwinner et al. (1983) observed that both DDE and VC gave equivalent binding to several rat tissues (Table 4.1), but only VC gave rise to measurable DNA adducts.

Guengerich, Mason et al. (1981) used epoxide hydrolase and alcohol dehydrogenase as inhibitors of adduct formation in reconstructed Sprague-Dawley rat liver microsomal oxidase systems and in hepatocytes exposed to vinyl bromide (VB) or VC. They observed that epoxide hydrolase inhibited DNA adduct formation, but not protein adduct formation from the vinyl halide metabolites, and that alcohol dehydrogenase inhibited protein adduct formation, but not DNA adduct formation. Reduced glutathione blocked protein adduct formation from CAA but did not block low level binding to DNA. The major form of

Table 4.1. Binding of CEO precursors to macromolecules *in vivo*[a]

Tissue	DDE	VC
Liver	0.32	0.32
Lung	0.07	0.08
Spleen	0.06	0.08
Kidney	0.17	0.11
Small intestine	0.12	0.09
Muscle	0.01	0.01

[a]Exposure of Wistar rats, by inhalation, to 0.12 mmole of VC over an 18 h period (Gwinner et al. 1983). Percent radioactivity bound irreversibly per g tissue. DDE is 2,2'-dichlorodiethylether, a unique precursor of CAA.

P-450 enzymes that oxidize VB and VC are not the same enzymes induced by phenobarbital or β-napthoflavone, although a significantly larger amount of hexane insoluble metabolites was formed on pre-exposure of the animal to these agents: 3-methylcholanthrene, Arochlor 1254, and 2-acetylaminofluorene. These results are consistent with a DNA adduct formation mechanism that requires reaction of an epoxide and not the haloacetalehyde rearrangement product with DNA. Exogenous DNA added to hepatocytes exposed to VC and VB contained significant amounts of adduct. This indicates that the epoxide's micro-lifetime was sufficient for it to escape the cell membrane and react at a distance from its site of formation.

4.2 ADDUCT FORMATION

4.2.1 *In Vivo* and *In Vitro* DNA and RNA Adducts

Exposure of Wistar rats to radiolabelled VC and DDE by inhalation results in essentially the same level of irreversible binding of radioactivity to liver, lung, spleen, kidney, small intestine, and muscle tissue (Gwinner et al. 1983) (Table 4.1). There was, however, no evidence for binding of DDE to RNA or DNA.

A number of different chemicals give rise to N^7-(2-oxoethyl)guanine 7-[OEG] and 2,3-(EG) after *in vivo* exposure (Table 4.2). Two bases derived from DNA adducts, 7-(OEG) and 2,3-(EG), have been identified after *in vivo* exposure to VC (Scherer et al. 1981, Green and Hathway 1978). Of the two bases, only the 2,3-EG has been demonstrated to exhibit characteristics of a mutagen. This was done by using a transcription based assay for introduction of errors using a ribocopolymer, made from 2.3-EG diphosphate and cytidine diphosphate

Table 4.2. Chemical sources of VC-related adducts

Chemical	Adduct[a]	Source	Reference
VC	7-(OEG)	*In vivo*	Scherer et al. 1981
VC	7-(OEG)	*In vivo*	Green and Hathway 1978
VC	7-(OEG)	*In vivo*	Osterman-Golkar et al. 1977
VC	7-(OEG)	*In vivo*	Gwinner et al. 1983
Ethylcarbamate	7-(OEG)	*In vivo*	Scherer, Steward, and Emmelot 1980
AN	7-(OEG)	*In vitro*	Guengerich et al. 1981
1,2-Dichloroethane	7-(OEG);3-EC	*In vivo*	Svensson and Osterman-Golkar 1986

[a]7-OEG, N^7-(2-oxoethyl)guanine; 3-EC, 3,N^4-ethenocytosine.

as template, and avian myeloblastosis virus reverse transcriptase synthesis of DNA (Singer et al. 1987). This observation and the observation by Barbin, Laib, and Bartsch (1985) that 7-(OEG) does not cause miscoding raises questions about the numerous suggestions that CEO

is the ultimate carcinogenic metabolite derived from VC. The DNA adduct that is responsible for initiation of carcinogenesis cannot be unequivocally defined at this time.

Several lines of evidence indicate that the cyclic nucleotides, 1,N^6-ethenoadenosine and 3,N^4-ethenoguanosine, are not formed on DNA *in vivo* but are formed on RNA *in vivo* and on DNA *in vitro* (Laib, Gwinner, and Bolt 1981; Gwinner et al. 1983). Significant adduct formation from CAA, *in vitro* and *in vivo*, is found only with proteins (Guengerich et al. 1981). The major adduct formed by VC *in vivo* is 7-OEG (Table 4.3) (Osterman-Golkar et al. 1977, Gwinner et al. 1983). A less conservative

Table 4.3. *In vivo* alkylation of DNA in rodents by VC[a]

Species/organ/treatment	Alkylated bases[b]			
	7-OEG	3-EG	1-EA	3-EC
Mouse/liver/single i.p. VC	8.3	-	(1)	n.d.
Rat/liver/250 ppm in H_2O for two years	-	-	trace	
Rat/liver/single exp to VC	4.5			
Rat/liver/2 exposures	5.2			
12 day old rat/liver/ 2 exposures	25.7	0.25		

[a]Adapted from Laib 1986 (Table I).
[b]7-OEG, N^7-(2-oxoethyl)guanine; 3-EG, 3,N^2-ethenoguanine; 1-EA, 1,N-6-ethenoadenine; 3-EC,3,N^4-ethenocytosine. Amounts are moles of modified base/10^6 mole unmodified base.

interpretation of the chromatographic separation of VC-DNA-adduct hydrolysates suggests that minor amounts of other adducts are also present when [^{14}C]-VC is the substrate (Gwinner et al. 1983).

These minor adducts amount to a substitution level of 1 adduct per 10^6 bases compared to > 50 7-OEG adducts per 10^6 bases (Gwinner et al. 1983). Special care was taken to minimize RNA contamination, and the minor adducts did not chromatograph in the position of 1,N^6-ethenoadenosine or 3,N^4-ethenocytidine. The positions for the corresponding etheno-deoxynucleosides were not described by Gwinner et al. (1983). Further evidence for the absence of etheno derivatives in

DNA was obtained by comparing adduct formation *in vitro* using DNA plus microsomes with adduct formation *in vivo* in male Wistar rats.

The hydrolytic stability of 7-alkylguanosine in RNA is much greater than the 7-alkyldeoxyguanosine derivative in DNA. Both break down to 7-OEG. Measurements of 7-OEG after incubation of DNA plus endonucleases and DNA plus phosphatases show essentially complete conversion of the putative N^7-(2-oxoethyl)deoxyguanosine (7-OEdG) to 7-OEG. The loss of 7-OEG from liver DNA, after exposure of 200 g male Wistar rats to 205 ppm VC for 5 h (23.4 μmol metabolized per rat), occurred with a half-life of about 57 h (Laib, Gwinner, and Bolt 1981) (Table 4.4). This is slower than the rate of repair of 7-OEG (half-life of ~20 h) derived from EO modification of DNA (see Sect. 2.0).

Table 4.4. Half-lives of DNA adducts derived from vinyl chloride exposure[a]

Animal	Tissue	Half-life
Male Wistar rat	Liver	57 h (as 7-OEG)[b]
Male Wistar rat	Liver	≈24 h (DNA radioactivity)[c]

[a]Exposure of the intact animal to VC vapors.
[b]Laib, Gwinner, and Bolt 1981b.
[c]Bolt et al. 1976.

N^2,3-ethenoguanine (2,3-EG) has been found as a minor rat liver NA adduct (Laib et al. 1985). The high level of fluorescence of the adduct permitted its detection. 2,3-EG is formed by reaction of CAA with guanosine (Oesch and Doerjer 1982). Recently this adduct has been observed in young rats (12 day old rats are more susceptible to VC induced carcinogenesis) (Laib et al. 1985). This observation indicates that the CAA-induced initiation of carcinogenesis is not yet ruled out.

Although double-stranded DNA does not accumulate VC metabolite-derived adducts at positions protected by H-bonding, the modified base 3,N^4-ethenocytosine in the template DNA does induce replication errors (Singer and Spengler 1986).

Bedell et al. (1986) have developed a sensitive and quantitative fluorescence method for detection of etheno adducts in DNA. They have observed the formation of N^2,3-ethenodeoxyguanosine, 1,N^6-ethenodeoxyadenosine, and 3,N^4-ethenodeoxycytidine from the reaction of CAA with calf-thymus DNA.

4.2.2 *In Vitro* Modification of Bases

The reaction of CEO with guanosine or deoxyguanosine derivatives in glacial acetic acid leads primarily to 7-OEG formation. Both the 7-alkyl nucleosides and free bases are highly fluorescent, with excitation wavelengths below 300 nm and an emission maximum near 370 nm, for the nucleoside derivative in 0.01 M HCl or in neutrality, and emission maxima at 340 nm, or 360 nm, for the free base at pH 10, or 2 N HCl, respectively. In 2 N NaOH, a maximum near 420 nm is observed (Scherer et al. 1981). Scherer suggests that reversible formation of a hydrated aldehyde and/or cyclic hemiacetal between the guanine O^6 position and the aldehyde group from the N^7-(2-oxoethyl) position occurs because of anomalous NMR proton shift assignments, anomalous mass spectrographic ion mass values, the instability of the 2,4-dinitrophenylhydrazone, and an unusually fast reaction of the compound with borohydride. CAA, which can be produced by rearrangement of CEO, reacts with deoxyguanosine and guanosine to give 1,N^2-ethenoguanine (1,2-EG) and 3,N^2-ethenoguanine (3,2-EG) (Laib 1986, Sattsangi et al. 1977, Oesch and Doerjer 1982).

The reaction of CEO or CAA with deoxycytidine or cytosine gives rise to 3,N^4-ethenocytosine (3EC) and 3,N^4-(N^4-hydroxy-ethano)cytosine, while the reaction of CEO and CAA with adenine or deoxyadenosine gives rise to 1,N^6 ethenoadenine (EA) and 1,N^6-(N^6-α-hydroxyethano)adenine (Barrio et al. 1972, Kotchetkov et al. 1971, Barbin et al. 1975, Guengerich et al. 1979). When the N^3 position of cytosine or deoxycytidine is blocked by methylation, then the favored products are <u>cis/trans</u> N^4-(2-chlorovinyl)-3-methylcytosine that may arise from an intermediate N^4-(1-hydroxy-2-chloroethyl)-3-methylcytosine (O'Neill et al. 1986) (Table 4.5).

4.3 STRUCTURE ACTIVITY RELATIONSHIPS

The toxicities of haloethylenes have been compared, and Henschler and his colleagues (see Bolt 1984) describe a concept according to which the haloethylenes with minimum chemical stability should have the highest mutagenic activity. They observed that symmetrically substituted polyhaloethylenes were less reactive than asymmetrically substituted isomers and that rearrangement to aldehydes or carboxylic acids also had to be taken into consideration. Bolt modified this hypothesis after comparisons of tumorigenic potential of several haloethylenes did not fulfill the hypothesis. Bolt reported that a balance between chemical reactivity and quantitative metabolism gave a better correlation between tumorigenicity and chemical structure. This idea was corroborated by molecular structure studies which compared the C-O bond energies to mutagenicity. Vinyl chloride showed the highest optimum stability value.

Scherer et al. (1981) suggest that the hypothetical cyclic hemiacetal structure of the adduct will alter the H-bonding properties of the O-6 oxygen of the guanine base so that normal base-pairing does not occur. The resultant sequence alteration is suggested to be the

Table 4.5. *In vitro* synthesis of VC-related adducts

Chemical reaction	Adduct	Reference
Chloroacetaldehyde plus DNA	3,N^2-ethenoguanine	Green and Hathway 1978
Chloroacetaldehyde with guanosine	3,N^2-ethenoguanine	Oesch and Doerjer 1982
Vinyl chloride plus RNA	1,N^6-ethenoadenosine	Laib and Bolt 1977, 1978
Chloroacetaldehyde plus homopolymers and DNA	3,N^4-ethenocytosine	Laib 1986
Chloroacetaldehyde plus homopolymers and DNA	1,N^6-ethenoadenine	Laib 1986
Chloroacetaldehyde plus homopolymers and DNA	3,N^4-(N-4-hydroxyethano) cytosine	Barbin et al. 1981
Chloroacetaldehyde plus homopolymers and DNA	1,N^6-(N-6-hydroxyethano) adenine	Laib et al. 1986.
Chloroethylene oxide plus deoxycytidine	3,N^4-(2(5),2-chlorovinyl)-ethenocytosine	Barbin et al. 1986
Chloroethylene oxide, Chloroacetaldehyde plus 3-methylde-oxycytidine	3-methyl,N^4-(2-chlorovinyl) cytosine (cis/trans isomers)	Barbin et al. 1986
Chloroethylene oxide plus methylcytidine	Multiple isomers at N4,C2, C3,C5 diadduct isomers	Barbin et al. 1986
Chloroacetaldehyde, Chloroethylene oxide plus 3-methyluridine	Ribosyl-1-hydroxy-2-chloroethyl derivatives	Barbin et al. 1986

basis for the carcinogenic action of VC. Barbin, Laib, and Bartsch (1985) tested this hypothesis by measuring the miscoding properties of VC adducts to poly(deoxyguanylate-deoxycytidylate) templates in a

replication fidelity assay using *E. coli* DNA polymerase I. They observed that about 80% of the mispairing events were located opposite minor cytosine lesions. They observed no mispairing opposite 7-OEG residues which comprised about 98% of the measurable adducts. They conclude that 7-OEG lacks miscoding properties.

Guengerich et al. (1981) observed that the adduct-forming metabolites of VC can traverse the cell membrane of hepatocytes and react with DNA and protein in the medium. They suggest that this may be an important parameter during initiation of the rare hemangiosarcomas since the endothelial cell targets have a lesser capacity to metabolize xenobiotics.

Ottenwalder et al. (1983) used exogenous activation of VC to demonstrate that cellular protein from rat hepatic sinusoidal cells, which includes the endothelial cells, can absorb externally generated CEO to form intracellular protein adducts. DNA adducts were not measured. They support the concept suggested by Guengerich et al. (1981) that the liver target cells that yield the hemangiosarcomas, and that do not express a vigorous xenobiotic metabolism, could be initiated by CEO diffusion from extra-sinusoidal cells.

Using *in vitro* assays for misincorporation of bases from templates containing modified bases, Singer and Spengler (1986) observed that 3-EC was capable of directing misincorporation for both transcription and replication type assays. They observed that the hydrated forms of 3-EC and 1-EA caused misincorporation in transcription assays but not in replication assays.

4.4 BIOLOGICAL ENDPOINTS

4.4.1 VC-Induced Base-Pair Miscoding

Studies of the miscoding induced by DNA adduct formation *in vitro* (Barbin and Bartsch 1986) and studies using synthetic CAA and CEO-modified templates indicated that miscoding events were not observed at 7-OEG positions but were observed opposite ethenocytosine positions (about 80% of observed errors), and these led largely to C:A mispairing. C:A mispairs were also generated by apurinic sites (Barbin and Bartsch 1986).

4.4.2 Carcinogenic Potency from DNA Adduct Levels

Bartsch et al. (1983) have developed mathematical correlations between the initial ratio of the levels of adduct formation on the N^7 guanyl position and the O^6-guanyl position in double-stranded DNA and the TD_{50} for rodents. When comparing these values for N-ethyl-N'-nitrosourea, N-methyl-N'-nitrosourea, N-methyl-N'-nitro-N-nitrosoguanidine, N-methyl-N-nitrosourethane, 1,3-propanesultone, β-propiolactone, glycidaldehyde, methylmethanesulfonate, epichlorohydrin,

and chloroethylene oxide, a linear regression was obtained with $r^2 = 0.86$. The following equation describes their results:

$$\ln(TD_{50}) = 1.394 \cdot \ln [N^7\text{-alkyl-G}/O^6 \text{ alkyl-G}] + 0.134 \qquad (4.1)$$

where TD_{50} is defined as the total dose of carcinogen in mg/kg bw required to reduce by one-half the probability of the animal being tumor free (at all sites) throughout a standard lifetime (Barbin and Bartsch 1986). The TD_{50} value for CEO was obtained from a mouse bioassay without a dose-response curve (quoted from Barbin and Bartsch 1986).

5. EPICHLOROHYDRIN

Epichlorohydrin (chloromethyl oxirane) (ECH) (CAS # 107-07-3) is used in the manufacture of epoxy resins, surface active agents, coatings, textiles, and insecticides. The permissible exposure limit (U.S.A) is 5 ppm (≈19 mg/m^3) (NIOSH 1985). ACGIH (1986d) recommends a lower limit of 2 ppm. Evidence for carcinogenicity is limited. There is an indication of tumor formation at the site of injection (C3H mouse) or lung papillomas after i.p. injection of ECH (Ehrenberg and Hussain 1981). Several studies demonstrate increased levels of chromosomal damage (Picciano 1979, Kucerova et al. 1977). In addition, some epidemiologic evidence exists for an increased incidence of birth defects in response to ECH exposure (Sram 1978, IARC 1985).

5.1 CHEMISTRY

Epichlorohydrin is a dense liquid at room temperature that boils at 117.9°C (760 mm Hg) and is insoluble in water. The presence of the chloromethyl group permits formation of three possible isomeric products from reaction with the oxirane group: one at the primary C-O position and a stereoisomeric pair at the secondary C-O position. Different proportions of products are formed depending on the solvent, bulkiness, and nucleophilic character of the nucleophile. In addition, the chloro group can also be replaced. The acid-catalyzed reactivity of ECH (through an intermediate carbonium ion) is reduced relative to EO or PO because of the electron-attracting properties of the chloromethyl group (Ehrenberg and Hussain 1981). The major path of reaction appears to be displacement with an SN2 type mechanism with predominant formation of a pair of isomeric secondary alcohol products (Hemminki and Lax 1986, Hemminki et al. 1980). The half-life for hydrolysis of the oxirane group by 0.1 M HCl at 37°C is 1 h (Ehrenberg and Hussain 1981).

5.2 METABOLISM

[^{14}C]-Epichlorohydrin is metabolized in rats via intermediate formation of α-chlorohydrin (ACH) (hydrolytic opening of the oxirane ring) and glycidol (You-fan and Hine 1986). The glycidol is believed to be formed by spontaneous hydrolysis of the chloromethyl group to an hydroxymethyl group. Rats converted radiolabelled ECH to CO_2 (38%) and excreted 50% into urine and 3% into feces. The remainder was bound in the tissue. The principal urinary products were N-acetyl-5(3-chloro-2-hydroxypropyl) L-cysteine (35%) and ACH (5%).

Epichlorohydrin has been suggested to be a metabolic product produced by *in vivo* metabolism of [^{14}C]-1,2-dibromo-3-chloropropane (DBCP) in rats (Laib 1982). Oral administration of 20 mg/kg bw DBCP to rats led to 99% recovery of the radioactive label in urine, bile, and expired air. Carbon dioxide was the main product in the expired air.

The mercapturic acids of S-(2,3-dihydroxypropyl) cysteine and 1,3(bis-cysteinyl)propan-2-ol, β-bromolactate and β-chlorolactate, have been identified in urine. Since ECH also gives rise to the two cysteinyl derivatives, the suggestion has been made that ECH is formed by dehydrohalogenation of DBCP.

Additional support for the metabolic conversion of ECH to detoxified substances is derived from studies that show the lifetime of ECH *in vivo* to be less than 20 min, while ACH injection shows residual ECH after 5 h of metabolism.

5.3 DNA ADDUCT FORMATION

5.3.1 *In Vitro* DNA Adduct Formation

The reactions of the epoxide group of epichlorohydrin with deoxynucleosides and commercial DNA preparations have shown the formation of covalent linkages with the N^7 position of deoxyguanosine and the N^6 position of deoxyadenosine, probably arising from the N^1 position of deoxyadenosine by the Dimroth rearrangement. The products of reaction with DNA are unknown but are presumed to be at the N^7 position as they are with most polar epoxides (Hemminki 1979). The reaction at the N^7 position of deoxyguanosine gives rise to three chromatographic products (see Table 5.1): two stereoisomers from attachment of the N^7 to the C-2 position and one peak which is believed to be a mixture of stereoisomers derived from depurination of the N^7-alkyldeoxyguanosinyl products. The depurinated product reacts further to form a diadduct at the N^9 and N^7 positions. The half-life of the N^7-alkyldeoxyguanosine derivatives toward alkaline-catalyzed ring opening

Table 5.1. Adducts formed from epichlorohydrin

Name	Source	Reference
N^7-±(3-chloro-1-hydroxy-2-propyl)deoxyguanosine	deoxyguanosine, DNA	Hemminki and Lax 1986
N^7-±(3-chloro-1-hydroxy-2-propyl)guanine	deoxyguanosine, DNA	Hemminki and Lax 1986
N^6-±(3-chloro-1-hydroxy-2-propyl)deoxyadenosine	deoxyadenosine, DNA	Hemminki 1979

at pH 8.5 and 28.5°C is 15 h, and the half-life for depurination at pH 5.5 is 3.4 h. The corresponding half-lives for the glycidol N^7-alkylguanine derivatives are 27 h and 4.4 h respectively (Hemminki and Lax 1986).

Intraperitoneal (i.p.) injection of 6.35 μmole/kg bw ECH into adult male Wistar rats or adult male BALB/c mice gave rise to DNA binding in liver, lung, kidney, and stomach in both sets of animals (Mazzulo et al. 1984) (Table 5.2).

Table 5.2. *In vivo* binding of radioactive ECH to rat and mouse organs[a]

	Level[b]			
	Liver	Kidney	Lung	Stomach
Rat	0.21	0.34	0.17	0.43
Mouse	0.14	0.11	0.06	0.23

[a]Exposure of 4 male Wistar rats and 12 BALB/c mice to 6.4 μmol/kg bw which is 200 fold lower than the LD_{50}. DNA was isolated 22 h post-exposure (Mazzullo et al. 1984).

[b]Levels given in pmol/10^6 pmol nucleotides in DNA. The levels of adducts in RNA and protein were about tenfold higher than for DNA.

5.3.2 DNA Damage Assays

Exposure of Hela cells or isolated mitochondrial DNA to 25 mM ECH for 4 min at 4°C results in significant cleavage of supercoiled DNA to the open circular and linear forms (Singh et al. 1985). The mechanism of cleavage was not described.

Indirect evidence that supports alkylation of the phosphate ester group in DNA leading to single strand breaks has been obtained by Solveig Walles and Ehrenberg (1968). The mechanism is believed to involve the formation of an RNA-like vic-diol ester linkage which is cleaved by alkali or heat. The single strand breaks occur in larger quantities than expected for simple depurination-induced strand breaks.

5.3.3 Estimation of Risk to Humans

Ehrenberg and Hussain (1981) have made estimates of exposures necessary to obtain a unit dose for several epoxides, including ECH. This exposure estimate is calculated using a relative potency comparison of gamma radiation and unit dose (M·h) of epoxide that gives the same mutation frequency in a standardized assay. This ratio is used

with radiation risk estimators to calculate human risk from epoxide exposure. The unit dose is a measure of the amount of DNA adduct formation. A unit EO dose was calculated to give a risk equivalent to 10 mrad-equiv [or (ppm·h)]. Epichlorohydrin was calculated to have a risk corresponding to ≈25 mrad-equiv [or (ppm·h)].

6. PROPYLENE OXIDE

Propylene oxide (methyloxirane) (PO) (CAS # 75-56-9) is used mainly as an intermediate in the production of polyether polyols (ingredients of polyurethane foams) and propylene glycol (IARC 1976b; Renne et al. 1986; Meylan et al. 1986). It is widely used as a fumigant for many foodstuffs and has been used as a sterilizing fumigant for plastic medical instruments and other materials (IARC 1976b). It is also used as a stabilizer for methylene chloride and fuel oils and for termite resistance treatment.

Current U.S. regulations specify an upper limit of 300 ppm for propylene oxide residue permitted in foods that will be further processed (CFR 21 Part 123.380); 700 mg/kg (as propylene glycol) is permitted in dried prunes and glazed fruits such as candied cherries (IARC 1976b). The OSHA's permissible level for worker exposure to propylene oxide is 100 ppm (NIOSH 1985), while ACGIH recommends a a TLV-TWA of 20 ppm (≈50 mg/m^3 for an 8 hr workday (NIOSH 1985).

Propylene oxide is a skin irritant in rabbits and an eye and nasal irritant in rats and guinea pigs, also causing drowsiness, weakness, and difficult breathing in the latter (Rowe et al. 1956). It has caused corneal burns in humans (McLaughlin 1946). It is mutagenic to yeast (Kolmark and Giles 1955), Drosophila (Rapoport 1948a,b; Schalet 1954), bacteria (Bootman, Lodge, and Whalley 1979) and phage (Garro and Phillips 1980); it induces chromosome damage in human lymphocytes *in vitro* (Bootman, Lodge, and Whalley 1979). Propylene oxide is carcinogenic to rats and mice, causing nasal epithelium and submandibular gland cancer in both species (Renne et al. 1986) and stomach cancer in rats (Dunkelberg 1982). In one study, it was tested and found negative for dominant lethal mutations in male mice (Bootman, Lodge, and Whalley 1979).

6.1 CHEMISTRY

Propylene oxide is a colorless liquid at room temperature. It has a low boiling point of 35°C and a freezing point of -112°C (Meylan et al. 1986). Propylene oxide is relatively miscible with water; it is also miscible with common organic solvents (Weast and Astle 1981-82). Propylene oxide in contact with water undergoes hydrolysis to yield 1,2-propylene glycol. *In vivo*, PO will react to yield glycols and halohydrins. Meylan et al. (1986) indicate that in fresh water at pH 7, the calculated hydrolysis half-life is 12.9 days (at 25°C); the combined calculated half-life of hydrolysis and reaction with Cl^- in sea water is 2.4 days.

The nucleophile 4-(p-nitrobenzyl)-pyridine has been used to measure rates of alkylation. Hemminki and Falck (1979) showed that this chemical, abbreviated 4NBP, reacts spontaneously with alkylating agents, acylating agents, and with nitrogen mustards, yielding a color change which is used for quantitative measurement of reactive groups. Hemminki et al. (1980) compared the reactivity of 4NBP with deoxyguanosine alkylation. They showed that PO was able to alkylate deoxyguanosine at 46% of the rate of epichlorohydrin.

6.2 ABSORPTION

Because PO is very reactive and has a short half-life (1 min at pH 1 at 37°C) along with a low molecular weight (58), Ehrenberg and Hussain (1981) suggest that it is radiomimetic in its dose distribution. Meylan et al. (1986) also mentioned that lung and gastrointestinal tract absorption can occur because of the higher water solubility and that bioaccumulation is not likely.

6.3 METABOLISM

Propylene oxide is formed by oxidative metabolism of propene in male CBA mice. Groups of 15 mice were exposed to 11 liters of propene gas at concentrations of 95, 250, 325, 380, 500, 780, 1500 and 1715 ppm. The rate of disappearance was monitored for at least one half-life. The rate curves of uptake yielded pharmacokinetic values of Km = 800 ± 60 ppm and Vmax = 8 ± 0.5 $kg^{-1} \cdot h^{-1}$ (Svensson and Ostermann-Golkar 1984).

6.4 DNA ADDUCT FORMATION

6.4.1 *In Vitro* DNA Adduct Formation

DNA adduct studies have been carried out *in vitro* and *in vivo* (Table 6.1). Deoxycytidine was alkylated by PO in 3 positions (O-2, N-3 and N-4) by Djuric and Sinsheimer (1984a). The same group also found that deoxythymidine was alkylated at the N-3 position (1984b). Hemminki et al. (1980) found deoxyguanine alkylated at the N-7 and deoxyadenosine alkylated at the N-6 position. Djuric et al. (1986) also found adenosine alkylated at the N-1 position.

Single-stranded DNA was found to form three types of adducts: guanine adducts (unidentified), N^3-(2-hydroxypropyl)adenine and N^7-(2-hydroxypropyl)guanine (Hemminki 1979; Lawley and Jarman 1972). Double-stranded DNA (calf thymus DNA [CT DNA] in all cases where the source was named) formed guanine adducts (Hemminki 1979), unidentified adducts in all four bases (Djuric et al. 1986) and an unknown adduct (Randerath, Reddy, and Gupta 1981).

6.4.2 Observed Binding of PO to DNA and Hb *In Vivo*

Propylene oxide is known to form hemoglobin adducts (hydroxypropyl-histidine adducts). These were observed by gas chromatography and mass spectrophotometry (Farmer, Gorf, and Bailey 1982; Farmer, Bailey and Skuker 1983).

Svensson and Osterman-Golkar (1984) attempted to measure the level of N^7-(2-hydroxypropyl)guanine in DNA from exposed mice. Based on the rate constants of formation for EO adducts in Hb and in DNA (assuming the values for EO and PO are identical), they estimated that at a given dose a low but measurable level of N^7-(2-hydroxypropyl) guanine adduct would be formed. The observed amount was less than calculated (none was observed).

Table 6.1. Propylene oxide adducts[a]

Name	Source	Reference
O^2-(alkyl[b])-deoxycytidine	Deoxycytidine	Djuric and Sinsheimer 1984[b]
N^3-(alkyl[b])-deoxycytidine	Deoxycytidine	
N^4-(alkyl[b])-deoxycytidine	Deoxycytidine	
N^1-(alkyl[b])-deoxyadenosine	Deoxyadenosine	Djuric et al. 1986
Unknown minor deoxyadenosine	Deoxyadenosine	
Unknown deoxyguanosine, (2 derivatives)	Deoxyguanosine	
Unknown[c]	CT DNA	
Unknown[d]	Mice	Svensson and Osterman-Golkar 1984
Unknown	DNA	Randerath, Reddy and Gupta 1981
3-(2-Hydroxypropyl)-adenine	Single-stranded DNA	Lawley and Jarman 1972
7-(2-Hydroxypropyl)-guanine	Single-stranded DNA	
Unknown	Single-stranded DNA	Hemminki 1979
7-(Alkyl[b])-guanine	Deoxyguanylic acid	Hemminki et al. 1980
N^6-(alkyl[b])-adenine	Deoxyadenylic acid	
3-(Alkyl[b])-thymidine	Deoxythymidylate	Djuric and Sinsheimer 1984[b]

[a]All identified adducts were isolated after reaction *in vitro*.

[b]The general term alkyl indicates that more than one isomer is present.

[c]No observable adducts were formed; evidence for adduct formation is based on mutagenesis data.

[d]Any adduct formed was below the limit of detection (40 femtomole/55mg DNA, based on a minimum detectable limit of 10 cpm with a specific activity of 119 Ci/mole).

7. 4,4'-METHYLENEBIS-2-CHLOROANILINE

The DuPont trade name for 4,4'-methylenebis-2-chloroaniline is MOCA (CAS # 101-4-4). MOCA is also known as MOCHA and as MBOCA, and it is structurally related to aniline. MOCA is used as a hardening agent and a curing agent in the polyurethane industry. Specifically, it is used in the manufacture of "integral-skin polyurethane semi-rigid foam" (which is used for crash padding) and objects like roller skate wheels. It has many characteristics of aromatic amines, and is mildly cyanogenic. It was also reported to be a kidney irritant (Van Roosmalen, Klein, and Drummond 1979). The current recommended TLV-TWA for MOCA is 0.02 ppm or approximately 0.22 mg/m^3 (ACGIH 1986f). The oral LD_{50} for rats is 750 mg/kg body weight (Farmer, Rickard, and Robertson 1981).

MOCA is mutagenic in the Ames Salmonella assay (Hesbert, Bottin, and DeCeaurriz 1985; Manis and Braselton 1984; Cocker et al. 1985). Because it is structurally related to aniline, MOCA is a suspect bladder and liver carcinogen (ACGIH 1986f). MOCA causes lung and liver tumors in the rat (Stula et al. 1975, Russfield et al. 1975) and is tumorigenic to mice (Russfield et al. 1975). It probably induces bladder tumors in dogs (Stula et al. 1977).

7.1 CHEMISTRY

MOCA is a solid that melts at approximately 100°C and breaks down at approximately 200°C. It can be held at 120°C indefinitely. To use it, MOCA must be mixed with an isocyanate before molding (Henning 1973). Two forms of MOCA are available. Originally, pellets of solid MOCA were melted and mixed at the site, but MOCA is now obtainable as a premix in which it is already dissolved in a polyol. The solid form is thought to lead to greater possibilities for worker exposure through adherence to workers' clothing.

7.2 METABOLISM

7.2.1 Human Metabolism

Absorption of MOCA takes place primarily through the skin but also can occur through breathing vapors of the melted compound. In humans, most absorbed MOCA is excreted in urine within 24 h (Thomas and Wilson 1984). The monitoring done by Thomas and Wilson showed a large variation in the urine concentration in the human population.

Linch et al. (1971) found two metabolites of MOCA, an N-mono- and an N-diacetyl MOCA in urine samples of humans exposed to MOCA in the workplace. These conjugates were not hydrolyzed by the esterase enzyme mixture used to prepare specimens for thin layer chromatography (TLC). The acid hydrolysis technique used to prepare the specimens for other

protocols also hydrolyzed the conjugates. The authors defined three classes based on urinary levels of excretion products: excreters (who excreted more than 2 mg of MOCA per liter of urine, and who required more than a month after the last exposure to clear MOCA from the urine), non-excreters (who excreted 0.5 mg/L or less, and who only required a week to clear MOCA from the urine) and intermediate excreters.

Linch et al. (1971) suggest that, because of its insolubility, the N,N'-diacetyl-MOCA derivative would probably be very hard to detect. However, Ducos, Maire, and Gaudin (1985) were able to find both MOCA metabolites in many human urine samples, although they only rarely found the diacetyl derivative.

7.2.2 Rat Metabolism

Rats excreted most of the label in the feces when given labeled MOCA i.p. Approximately 60% of the rest was excreted in the urine, although only 1-2% was present as the parent compound (Farmer, Rickard, and Robertson 1981). The liver retained some of the label as did adipose tissue (white fat). Of the excreted MOCA, some of the metabolites were determined to be conjugates which were hydrolyzed by glucuronidase and sulphatase. However, in human urine, the concentration of unmodified MOCA was much lower than in rats, and no evidence was found for either of the major conjugated metabolites previously found in rat urine. Dermal administration of MOCA to rats results in approximately 50% excretion within 72 h (Ward et al. 1986).

7.2.3 Dog Metabolism

Manis and Braselton (1984) found that the major metabolite in dogs is a conjugate sensitive to arylsulfatase. This metabolite is 5-hydroxy-3,3'-dichloro-4,4' diaminodiphenylmethane-5-sulfate. However, they did not find this hydroxylsulfate in the urine of exposed human workers.

7.3 DNA ADDUCT FORMATION

There is only one published work indicating any type of direct DNA binding by MOCA or its metabolites. Manis and Braselton (1984) found that the hydroxylated sulfate conjugate, incubated with arylsulfatase and DNA, could bind in 24 h to a maximum of 52 pmol/mg DNA. They also found some capability to bind to protein. Farmer, Rickard, and Robertson (1981) questioned whether the retention of labeled MOCA in the liver might be due to the covalent binding of reactive metabolites, but nothing published on the subject has been found.

7.4 DNA DAMAGE STUDIES

In another type of assay, McQueen et al. (1981) showed that MOCA is genotoxic to mouse, rat, and hamster primary hepatocytes. Addition of MOCA to hepatocytes caused the cells to incorporate ^{3}H-TdR, indicating damage to the DNA of all three species. In a later study, McQueen, Maslansky, and Williams (1983) demonstrated that MOCA caused weakly positive results in hepatocytes from some, but not all, rabbits tested. The method again involved monitoring ^{3}H-TdR incorporation by quantitative autoradiographic techniques.

8. BENZIDINE AND BENZIDINE DYES: DIRECT BLUE 6, DIRECT BLACK 38, AND DIRECT BROWN 95

Direct Black 38 (DBK) (CAS # 1937-37-7), Direct Brown 95 (DBR) (CAS # 16071-86-6), and Direct Blue 6 (DBU) (CAS # 2602-46-2) are used in a variety of industries including: paper, paper-products, petroleum and related industries, rubber and plastic products, leather and leather products, instrumentation, measuring devices, the banking industry, and the textile industry (Thomas et al. 1978). Each of these dyes is prepared by the condensation of benzidine (BZ) (CAS # 92-87-5) with water soluble amino-napthalenesulfonic acids and amino-benzene derivatives. All three dyes have been shown, in preliminary short-term feeding studies, to be carcinogenic in rats and mice (Robens et al. 1980). These animals all excrete significantly larger quantities of BZ than would be expected from the trace quantities present in the food. Similar observations have been made in exposed humans (IARC 1982c,d,e). The conclusion is that the absorbed dyes are catabolized to BZ prior to detoxification. It is believed that the observed cancers were produced from BZ produced by cleavage and reduction within the liver and probably not by cleavage and reduction by intestinal flora. An additional carcinogenic mechanism should also be considered: the human reduction of BZ to 4-aminobiphenyl (Cerniglia et al. 1986). IARC (1982c,d,e) has concluded that occupational exposure to benzidine-based dyes constitutes a carcinogenic risk to humans. Even where these dyes are used, stringent industrial hygiene measures can afford good worker protection. For example, in a leather tannery where good work practices were observed, no BZ was observed in worker urine.

Benzidine is a urinary bladder carcinogen in humans and dogs (IARC 1982b) and is a hepatocarcinogen in rodents such as rats, mice, and hamsters (Beland and Kadlubar 1985). Other sites of tumor formation include mouse Harderian gland and lymphoreticular system, and the Zymbal's gland, mammary gland, and intestine of rats (Morton, King, and Baetcke 1979). Benzidine is regulated as a human carcinogen by OSHA; all exposures are to be kept to an absolute minimum. There is no TLV for benzidine (ACGIH 1986b).

8.1 CHEMISTRY

The oxidation of benzidine by reactions mediated by free radicals results in the formation of several highly reactive electrophilic species which undergo a variety of reactions including spontaneous polymerization, and formation of conjugates with thiols, and with phenols. The thiols, glutathione, cysteine, and N-acetylcysteine, all react with oxidized benzidine to form conjugates. The N-acetylcysteine product is an S-3 substituted benzidine (Josephy 1985). In the absence of an excess of thiol, the oxidized benzidine is reversibly reduced back to benzidine.

8.2 METABOLISM

8.2.1 Metabolism of the Dyes

The benzidine-diiminyl structures in DBK, DBR, and DBU are obtained by oxidation of both amino nitrogens followed by condensation into diazo- linkages (Garner, Martin, and Clayson 1984). Thus the formation of free BZ from the three dyes would require an intermediate reductive step as well as a hydrolytic step.

This concept of prior reduction has been incorporated into several mutagenicity studies in which the dye has been pretreated with either chemical reduction (dithionate, dithionite) or enzymatic reduction prior to testing (Gregory, Elliot, and Kluge 1981; Cerniglia et al. 1986; Prival et al. 1984; Martin and Kennelly 1981; and Joachim, Burrell, and Anderson 1985). In general, prior reduction did increase mutagenicity with congeners of mutagenic bases.

Cerniglia et al. (1986) used freshly voided human feces to demonstrate anaerobic reduction of DBK 38 which resulted in the formation of BZ, monoacetylbenzidine (ABZ), 4-aminobiphenyl, and 4-acetylaminobiphenyl derivatives. Martin and Kennelly (1981) used phenobarbitone-induced male Wistar rats to test liver azoreductase activity in forming reduced aromatic amines and in inducing mutagenicity from azo dyes. They suggest that the observed mutagenicity may also be due to the formation of 1,2,4-triaminobenzene derived from reduction of DBK 38.

Kennelly, Hertzog, and Martin (1982) suggest that the carcinogenic properties of DBK, DBR, and DBU result from reduction within the liver since other dyes found not to be carcinogenic (Congo red, trypan blue, and Chicago sky blue) were also not reductively metabolized by rat liver. All the dyes, carcinogenic and non-carcinogenic, are reductively metabolized by intestinal microflora.

A counterpoint to the suggested need for reduction by added reducing agents is the report by Yamazoe, Roth, and Kadlubar (1986) that free benzidine diimine (BZDI), which has the same electronic structure as the condensed BZ in the dyes, can undergo an internal rearrangement which results in the BZ arylnitrenium intermediate. This intermediate is believed to be the chemically reactive species which forms the major DNA adduct. Thus, only hydrolysis of the dye is required to provide the "ultimate carcinogenic" form of BZ. This does not explain the apparent liver catabolism specificity for the three dyes, unless the liver has a specific hydrolase capable of forming significant quantities of BZDI.

8.2.2 Metabolism of Benzidine

Benzidine is readily absorbed both upon inhalation and skin exposure (ACGIH 1986b). Nonexposed humans show no detectable level of benzidine in urine (limit of detection [LOD] = 2 μg/L) (Van Sittert and De Jong 1985). Workers in contact with DBK have been shown to excrete BZ as well as 2,4-diaminoazobenzene (DiAmAzBz) (Lowry et al. 1980).

Liver perfusion studies with BZ and male Fischer 344 rats (Lynn et al. 1983) demonstrated that BZ is converted to N-glucuronidyl-BZ (GBZ), N-acetylbenzidine (ABZ), and to N,N'-diacetylbenzidine (DABZ). Subsequent metabolism led to the oxy-glucuronide of DABZ as the major end product of metabolism. Twenty-eight different radioactive derivatives of BZ were observed in the perfusates (identities not listed) and included the 3-O-glucuronide of DABZ. A similar distribution of metabolites was demonstrated with *in vivo* injection of BZ (Lynn et al. 1984). Sulfur-containing metabolites were also observed in the *in vivo* perfusion study (Lynn et al. 1983). Diacetylbenzidine was deacetylated, and N-OH-DABZ was rapidly reduced when added to the perfusion fluid.

In a pair of papers, Morton, King, and Baetcke (1979) and Morton et al. (1980) suggested that the diacetylbenzidine (DABZ) metabolite N-hydroxy-N,N'-DABZ was the more likely form of the ultimate carcinogen of BZ, based on *in vitro* studies using hepatic cytosol, and that the hepatic sulfotransferase was the activating step. In a study of the comparative carcinogenic properties of BZ and N-OH-DABZ, Morton et al. (1981) found that the N-hydroxy-DABZ was highly toxic (70 μmole/kg bw was lethal). Chronic lower doses (10 or 30 μmol/kg bw over 46 weeks) showed low toxicity. Benzidine and DABZ showed about the same level of carcinogenicity while the N-OH-DABZ achieved that level of carcinogenicity at one-third the dose. The corresponding monoacetyl derivatives were not included in this study.

Mammalian oxidative enzymes have been demonstrated to catalyze BZ oxidation by two mechanisms: liver NADPH (NADH) dependent microsomal oxidases and arachidonic acid dependent peroxidase (Frederick et al. 1985, Josephy 1985). NADPH dependent microsomal oxidation by adult rodents (male Nctr:S23[SD] and B6CF1/Nctr mice) hydroxylate both ABZ and DABZ, but the rate of hydroxylation of both nitrogens of ABZ is faster than the oxidation of DABZ. The formation of the hydroxylamine of ABZ (i.e. N-OH,N'-Acetyl-BZ) was faster than the formation of the hydroxamic acid of ABZ (i.e. N-OH,N-Acetyl-BZ) in both species. Acetylation rates with these enzyme preparations were about three to ten times faster than the oxidation rates (acylation 0.7 to 0.29 nmol/min/mg cytosolic protein) (Table 8.1). Deacetylation occurred at rates comparable to acetylation in mice but was only 1% to 2% of the rate of acetylation by rat liver cytosol. Frederick et al. (1985) suggest that the cytotoxic N-OH-DABZ is formed by the reversible acetylation of N'-OH-ABZ. The activation of the N-OH group to the reactive oxyester occurs with N,O-acyltransferase and N'-OH-ABZ cytosolic sulfotransferase. The chemical mechanism responsible for most reactions with benzidine involves intermediate formation of an aryl-nitrenium ion which reacts with nearby nucleophiles. The intermediate is formed from cleavage of the N-OR ester linkage.

The peroxidase-catalyzed oxidation of benzidine results from the combined action (cooxidation) of the peroxidase on either added hydrogen peroxide or organic peroxide (Josephy 1985). For example, the blood benzidine test is performed by addition of H_2O_2 to blood that contains endogenous peroxidases or methemoglobin; or benzidine is oxidized by the

Table 8.1. Rates of N-oxidation of acetylated BZ metabolites by mouse and rat liver microsomes[a]

Substrate	ABZ	DABZ	
Product	N-OH-ABZ	N'-OH-ABZ	N-OH-ABZ
Species			
mouse	0.19 ± 0.04	0.92 ± 0.08	0.26 ± 0.01
rat	0.11 ± 0.01	0.13 ± 0.0	0.005 ± 0.001

[a]The rates are expressed as nmol product/min/mg microsomal protein ± SD (37°C). Adapted from Frederick et al. 1985.

combined action of glucose oxidase, which produces H_2O_2 and a peroxidase; or benzidine is oxidized by the action of prostaglandin H synthase which converts arachidonic acid to an organic peroxide through the addition of oxygen. In the presence of excess N-acetylcysteine, peroxidase action on benzidine gives rise to 3-(S-(N-acetyl)-cysteine)-benzidine (Josephy and Iwaniw 1985).

8.3 DNA ADDUCT FORMATION

8.3.1 Adducts Induced by Benzidine Dyes

Formation of the adduct derivative, N-dG-N'-BZ, has been observed with the *in vitro* reaction of benzidine diimine (BZDI) with calf thymus DNA (Yamazoe et al. 1986). The mechanism of action is suggested to involve rearrangement of the monoprotonated form to the BZ-nitrenium ion which then condenses with the C-8 position of a deoxyguanosinyl residue in DNA.

Injection (i.p.) of 61.2 mg/kg bw of DBU or 76.7 mg/kg bw of Congo red into male Wistar rats gave rise to N-(deoxyguanosin-8-yl)-N'-acetylbenzidine (N-dG-N'-ABZ) after enzymatic hydrolysis of liver DNA (Kennelly, Shaw, and Martin 1984). As a control, 25 mg/kg bw of [^{3}H-acetyl-ABZ] was injected (i.p.) into other rats. The majority of the radioactive adduct from DBU injection was water soluble (i.e. was not extracted into n-butanol). The only adduct observed from ABZ or Congo red injection was N-dG-N'-ABZ. As shown in Table 8.2, the amount of binding to liver DNA decreased almost threefold between the first and seventh day post exposure in both ABZ and Congo red-treated rats, while the binding in DBU-treated animals increased by a factor of about three

Table 8.2. Persistence of DNA adducts[a]

Chemical	Day 1	Day 7	Adduct
ABZ	70	25	N-dG-N'-ABZ
DBU	8	22	N-dG-N'-ABZ + N-dG-N'-BZ + *b*
Congo-red	15	5	N-dG-N'-ABZ

[a]The values are in pmoles/mg DNA; from Kennelly, Shaw, and Martin 1984.

[b]Tentatively identified as the free base form and an adduct containing the benzidinyl and one of the amino-naphtholsulfonic acid residues (see following text).

between the first and seventh day after exposure. The level of binding was very high.

Between 75 and 85% of the radioactive nucleosides from the DBU-exposed liver DNA were present as the water-soluble compound. This material precipitated with the DNA hydrolases at the end of the digestion. Treatment of the water-soluble fraction and the protein precipitate with dithionite transformed the adduct to an n-butanol-soluble product. On chromatography, three major peaks were observed: a polar fraction which did not adsorb to the reversed phase column, N-dG-BZ, and an unidentified product which did not chromatograph at the positions for BZ, N-dG-N'-ABZ, or N-(deoxyguanosyn-8-yl)-N,N'-diacetylbenzidine (N-dG-N,N'-DABZ). The recovery of N-dG-BZ as a component of the water-soluble adduct after reduction indicates that the chemically reactive species contains the 4(4')-N-oxy group which can result in coupling to the C-8 of guanine. The authors suggest that only one of the two amino-naphtholdisulfonate groups of DBU remains attached to the benzidinyl residue. No chemical evidence is adduced to support the conclusion.

In an earlier study, Martin et al. (1983) compared the DNA adducts formed after administration of BZ, ABZ, DABZ, and DBU either orally or intraperitoneally. The BZ, ABZ, and DABZ studies were done with male Sprague-Dawley rats, and the DBU was done with a male Wistar-derived strain of rats (Bantin and Kingman Ltd. of Hull, UK). As shown in Table 8.3, the only adduct observed after administration of BZ, ABZ, or DABZ was the monoacetylated hydroxylamine-derived structure N-(deoxyguanosin-8-yl)-N'acetylbenzidine (>95%). The route of administration has a significant effect (Martin et al. 1983); intraperitoneal injection of DBU gave increasing levels of adduct formation over an interval of one week (an early peak at 8 h post exposure of 1589 nmol/mol DNA-P,

followed by an increase to 7656 nmol/mol DNA-P at 7 d). Oral administration gave a continuous increase from 12 nmol/mol DNA-P at 2 h to 111 nmol/mol DNA-P at 7 d.

Table 8.3. DNA adducts formed from benzidine and benzidine dyes, and benzidine derivatives

Chemical	Adduct	Reference
Benzidine	N-(deoxyguanosin-8-yl)-N'-ABZ	Martin et al. 1982
DBU	N-(deoxyguanosin-8-yl)-BZ N-(deoxyguanosin-8-yl)-N'-ABZ	Martin et al. 1983
	N-(8-guanyl)-BZ[b] N-(deoxyguanosin-8-yl), N'-N(1-naphthol-3, 6-disulfonate-imin-2-yl) BZ[b]	Kennelly, Shaw and Martin 1984
Benzidine-diimine	N-(deoxyguanosin-8-yl)-BZ[c]	Yamazoe et al. 1986
N-acetylbenzidine	N-(deoxyguanosin-8-yl)-N'-ABZ	Kennelly, Shaw, and Martin 1984
N,N'-diacetyl-benzidine	N-(deoxyguanosin-8-yl)-N'-ABZ	Martin et al. 1982, Kennelly et al. 1984

[a]Isolated from DNA after enzymatic digestion of DNA; formed *in vivo* unless otherwise indicated.
[b]Tentative identification, since no data are presented to support the structure.
[c] *In vitro* study.

Two adducts are observed with DBU: N-(deoxyguanosin-8-yl)-BZ (70%) and a tentatively identified derivative of that adduct without the deoxyribose portion N-(guan-8-yl)-benzidine (30%). There was no indication that reduction with dithionite was needed to produce the N-dG-N'-BZ.

8.3.2 Benzidine-Derived Adducts

Studies with rats, mice, hamsters, and dogs have shown that the monoacetylated N-dG-N'-ABZ is the primary DNA adduct formed *in vivo* from

injection of either BZ, ABZ, or DABZ (Martin et al. 1982, Kennelly et al. 1984, Frederick et al. 1985, Martin and Ekers 1980). The formation of the adduct of N-dG-N'-ABZ from DABZ occurs through removal of one of the acetyl groups, either before or after N-hydroxylation to the reactive intermediate. This equilibration of the several precursors with production of a single final adduct is one likely mechanism consistent with observed *in vivo* DNA adduct formation. In one set of experiments using a very high dose of 100 mg/kg bw, 15% of the liver DNA adducts had the diacetylated structure N-dG-N,N'-DABZ. The persistence of N-dG-N'-ABZ in this experiment was at least 28 days (Martin and Ekers 1980).

In vitro studies with the hydroxylamine and hydroxamic acid forms of ABZ and DABZ and DNA gave rise to the same DNA adduct derivative with the N-acetyl and N-guanyl substituents on opposite ends of the benzidinyl group (Martin et al. 1982). Peroxidase-catalyzed binding of BZ results in DNA adduct formation (Fourney, O'Brien, and Davidson 1986; Tsuruta, Josephy et al. 1985; Tsuruta, Subrahmanyam et al. 1985). Fourney, O'Brien, and Davidson (1986) added metabolites formed from BZ, horseradish peroxidase, and hydrogen peroxide to pBR322 DNA and observed a change in its state of aggregation as demonstrated by its inability to penetrate an 0.8% agarose gel. Tsuruta's group demonstrated binding of BZ/horseradish peroxidase derivatives to the four homoribonucleotide polymers (Tsuruta, Josephy et al. 1985; Tsuruta, Subrahmanyam et al. 1985). Poly-G, Poly-I, and Poly-X efficiently bound the oxidized benzidine derivatives (26, 25, and 31 nmol/mg, respectively). Poly-U was less efficient (7 nmol/mg). The level of binding with calf-thymus DNA was 30 nmol/mg. The identity of the adduct(s) is not known.

8.4 DNA DAMAGE STUDIES

High dose studies (500 to 1000 mg/kg) showed DBK to be a weak inducer of unscheduled DNA synthesis in male Wistar rat liver and bone-marrow micronuclei (Beije 1987). Similar high dose studies with DBR (100 mg/kg) also showed induction of unscheduled DNA synthesis. The latter assays were performed on isolated hepatocytes from animals that were either exposed or not exposed before isolation of the hepatocytes (Joachim and Decad 1984).

9. ACRYLONITRILE

Acrylonitrile (AN) (CAS # 107-13-1) is used in the manufacture of acrylic fibers and plastics and is an intermediate in the synthesis of some antioxidants, pharmaceuticals, and dyes (Duverger-Van Bogaert et al. 1981). The amount of AN produced in the United States has been estimated to be approximately 10^9 g/yr. The population of workers exposed to AN may be as large as 125,000. The current OSHA standard is 2 ppm (8-h TWA); the 15 min ceiling limit is 10 ppm (ACGIH 1986a).

Acrylonitrile is considered to be a probable human carcinogen (IARC 1982a) based on limited evidence of carcinogenicity of the CNS in rats (Koerselman and van der Graaf 1984) and on limited evidence for lung and stomach cancer in humans (Koerselman and van der Graaf 1984, ACGIH 1986a). Acrylonitrile and some of its metabolites are mutagenic in the Ames Salmonella assay; AN is also mutagenic to *E. coli*, yeast, Drosophila, and Chinese hamster ovary cells (Lambotte-Vandepaer and Duverger-Van Bogaert, 1984). It has tested negatively for chromosomal effects in studies of mouse bone narrow chromosome aberrations, the micronucleus test, and the dominant lethal test (Lambotte-Vandepaer and Duverger-Van Bogaert 1984). Some evidence suggestive of possible teratogenicity in rats and hamsters is reviewed by Lambotte-Vandepaer and Duverger-van Bogaert (1984); a study in chick embryos was negative (Kankaanpaa et al. 1979), and a review by the Council on Scientific Affairs of the AMA (1985) reports no evidence for maternal or fetal reproductive toxicity.

9.1 CHEMISTRY

Acrylonitrile is a liquid at room temperature, with a freezing point of -83.5°C and a boiling point of 77.3°C. The flash point is 32°F, and it is listed as being flammable with the capability to produce an explosive mixture in air at 25°C.

It is miscible with organic solvents, but only 7.3 parts of AN are miscible with 100 parts of water. The oral LD_{50} for rats is 0.093 g/kg. Acrylonitrile and its epoxide metabolite, 2-cyanoethylene oxide (CNEO), are both very reactive species that are able to undergo a variety of different routes of reaction and metabolism.

9.2 METABOLISM

9.2.1 *In Vitro* Microsome/Acrylonitrile Interactions

An examination of the spectral response of cytochrome P450- and P448-dependent hepatic microsomal oxidase preparations when incubated with AN (Appel et al. 1981) was aimed at understanding species-dependent contradictory results in formation of an intermediate epoxide. Human, rat, and mouse microsomes were compared. A ligand-binding type of

interaction was found with both human and rat microsomes. Mouse microsomes differed, showing a ligand-binding type interaction when benzo-(a)-pyrene-induced microsomes were used, but showing a Type I substrate-binding interaction when phenobarbital-induced mouse microsome preparations were used. Appel et al. concluded that human and rat microsomes behave similarly but differ from mouse microsomes.

9.2.2 *In Vivo* Metabolism Studies

Further work on the differences between mouse and rat metabolism of AN was done by Ahmed and Patel (1981). Because the toxicity of AN was believed to be due to release of cyanide, they compared the responses of mice and rats to AN and KCN. They used doses ranging from 0.25 to 2 times the LD_{50}, (the LD_{50} is 93 mg/kg for rats [male Sprague Dawley] and 27 mg/kg for mice [male albino Swiss]). The concept tested was that both species should have shown similar responses if the toxic metabolite were the same for both species. Mice given AN showed the same symptoms as mice and rats given KCN, but rats had a different response to AN. Rats exhibit an early cholinomimetic response, while mice exhibit CNS responses. This species difference in response is extended to the levels of cyanide in blood and tissues. While AN-treated rats had a lower concentration of CN in their blood and tissue than the KCN-dosed rats, there was no significant difference in the blood and tissue cyanide concentrations in AN-treated mice.

Examination of the role of glutathione (GSH) in the differences between rats and mice (Cote, Bowers, and Jaeger 1984) showed that GSH (which can react with several metabolites, as well as directly with AN) decreased the most in rat tissues, less with Syrian Golden hamster and mice. The LD_{50} values for these species are 100 mg/kg (rats), 60 mg/kg (hamster), and 30 mg/kg (mice).

Farooqui and Ahmed (1983b) found that GSH levels decreased by more than 85% within an hour of *in vitro* incubation of rat red blood cells with AN. S-cyanoethylglutathione is the major product and is stable in the intact erythrocyte. If the concentration of AN is high enough (0.05 M), the depletion of GSH results in the conversion of Hb to methemoglobin. These cells also become osmotically fragile and lyse.

9.2.3 Urinary Metabolites

Two different cysteine derivatives have been found in rat urine, N-acetyl-S-(2-hydroxyethyl)cysteine and N-acetyl-S-(2-cyanoethyl)cysteine, after exposure to doses ranging from 0.01 to 0.25 mmole per 200 g Wistar rats (van Bladeren et al. 1981). The cysteine derivative N-acetyl-S-(2-hydroxyethyl)cysteine in rat urine was not found after pretreatment of rats with a P450 inhibitor, while the N-acetyl-S-(2-cyanoethyl)cysteine is found in higher amounts. The two compounds are expected to have been formed by independent pathways: a direct chemical addition of AN to GSH to form N-acetyl-S-(2-cyanoethyl)cysteine and a metabolic oxidation of AN to form CEO which then reacts with the epoxide and loses the HCN group. A ratio of 72

(cyanoethyl) to 28 (hydroxyethyl) present in non-treated rat urine indicates that both pathways operate in the normal rat.

Langvardt et al. (1980) established that the cyano group of AN can be converted to thiocyanate prior to excretion and that a significant amount of the N-acetyl-S-(2-cyano-2hydroxyethyl)cysteine may be cyclized to N-acetyl-3-carboxyl-5-cyanotetrahydro-1,4-2H-thiazine. These were identified by ion exclusion chromatography and liquid chromatography, gas chromatography/infra-red spectrophotometry and/or gas chromatography/mass spectrophotometry. They found that a dose of 30 mg/kg (given in water) led to the excretion of 47% of the labelled dose within 16 hours. The thiazine derivative and thiocyanate are proposed to arise from 2,3-epoxy-propionitrile formed by oxidative metabolism of AN and were present in concentrations at least as high as N-acetyl-S-(2-cyanoethyl)cysteine.

9.2.4 2-Cyanoethylene Oxide

In addition to the study of urinary metabolites, the basic studies by F.P. Guengerich (Guengerich, Geiger et al. 1981; Geiger, Hogy, and Guengerich 1983; and Hogy and Guengerich 1986) explored the metabolism of AN in rats by using subcellular fractions (both rat and human microsomes), isolated rat hepatocytes, and rats *in vivo*. They found that CNEO is produced by microsomal enzymes. By using [^{14}C]-AN, they found that approximately 5% of the AN is bound to protein as S-(2-cyanoethyl)cysteinyl residues, and 80 to 90% of the total dose is found as S-(2-cyanoethyl)glutathione. They found that approximately 10% is oxidatively metabolized to CNEO; the subsequently released cyanide is converted to thiocyanate.

Lambotte-Vandepaer, Duverger-Van Bogaert, and Rollman (1985) exposed rats that had been pretreated with $CoCl_2$ (a P450 inhibitor) to 30 mg/kg AN i.p. They observed the expected decrease in urinary N-acetyl-S-(2-hydroxyethyl)cysteine concentration and an increase in N-acetyl-S-(2-cyanoethyl) cysteine concentration. Phenobarbital, which induces the P450-mixed function oxidase system, has the same effect. The authors suggest that this effect may also arise from an altered balance of detoxification reactions that would reduce the AN available for oxidative metabolism, and hence, detoxification of AN as well. Trichloroacetonitrile (a radical-trapping agent and mono-oxygenase inhibitor) did the same. Diethylmaleate (a glutathione depletor) decreased all three metabolite concentrations. They found a somewhat different distribution of metabolites from that observed by Guengerich; the i.p. and orally dosed rats had approximately 20% of the dose (measured by ^{14}C-labeled AN and identified by mass spectrometry) excreted as N-acetyl-S-(2-hydroxyethyl)cysteine and 15% as N-acetyl-S-(2-hydroxyethyl)cysteine (measured by gas-liquid chromatography and identified by gas/chromatography/mass spectrometry). Thiocyanate excretion varied according to the route of exposure. It was 22% after oral administration and only 3.7% after i.p. administration. In addition, mice given the same i.p. dose (relative to size) had similar levels of N-acetyl-S-(2-cyanoethyl) and (2-hydroxyethyl) cysteines but a

higher percentage of SCN^-(15%) indicating different metabolic pathway usage for the two different species.

Information on which metabolites may be produced *in vivo* has also come from work done *in vitro*. By incubating variously induced rat liver S9 preparations with AN, Duverger-van Bogaert et al. (1981) were able to isolate and identify four *in vitro* metabolites. These were glycolaldehyde (CH_2OH-CHO), cyanoacetic acid (NC-CH_2-COOH), 3-hydroxypropionitrile (HO-CH_2-CH_2-CN) and acetic acid (CH_3COOH). The glycolaldehyde probably forms by hydrolysis of the cyanohydrin formed from hydration of CNEO.

9.3 MACROMOLECULAR ADDUCT FORMATION

9.3.1 *In Vivo* DNA Adducts

In searches for DNA adducts, Fischer 344 rats (Hogy and Guengerich, 1986), Sprague-Dawley rats (Farooqui and Ahmed, 1983a), and Wistar rats (Peter, Appel et al. 1983) were exposed to AN in doses ranging from 0.2 mM (Peter, Appel et al. 1983) to 46.5 and 50 mg/kg (Table 9.1).

Table 9.1. Adducts formed from acrylonitrile *in vivo*[a]

Chemical	Dose	Liver	Brain	Stomach
	N^7-(2-oxoethyl)guanine[b]			
AN[b]	50 mg/kg bw	0.03	0.004	
CNEO[b]	6 mg/kg bw	0.014	0.0009	
	Unknown adducts (Sprague-Dawley rats)			
AN[c]	47 mg/kg bw	5.1	56.4	48.1
	Unknown adducts (Isolated rat hepatocytes)			
AN[d]		3		

[a]Adducts per 10^6 bases using radioactive detection.
[b]From Hogy and Guengerich (1986).
[c]From Farooqui and Ahmed (1983a); oral administration for 1 to 48 hr to Sprague-Dawley rats.
[d]From Geiger et al. (1983); 2 μM AN incubated with isolated rat hepatocytes and CT DNA in medium; no adducts found in hepatocyte DNA.

Peter, Appel et al. looked specifically for 1,N^6-ethenoadenosine and for 3,N^4-ethenocytidine. They found neither. However, two putative but unidentified alkylation peaks were found. Farooqui and Ahmed (1983a) found an unknown alkylation product. They found the maximum label bound at 24 h post-oral-dosing. The highest concentration of label was found in the brain, then the stomach and liver. The dose was approximately half the oral LD_{50} for rats, or 46.5 mg/kg. The binding, in μmol bound per mole DNA, was: for the brain, 56; for the stomach, 45; and for the liver, 5 (Table 9.1). Hogy and Guengerich (1986) were looking for 1,N^6-ethenoadenosine but found N^7-(2-oxoethyl)guanine instead.

9.3.2 *In Vitro* Binding to DNA and RNA

Guengerich, Geiger et al. (1981) found that the presence of NADPH enhanced the binding of radioactively labeled AN to calf thymus DNA. Binding occurred in the absence of NADPH as well. Light, which has been found by some workers to be required for mutagenicity, was not required for the DNA binding to occur. Human liver microsomes showed very little ability to enhance binding to DNA (only one of six different preparations showed any activity), but the rat liver microsome preparations did. In the presence of CNEO and rat liver microsomes, it was possible to show binding of CNEO metabolites to calf thymus DNA (2 mg DNA, phosphate buffer, pH 7.7, 0.25 mM AN or CNEO for 0 to 4 h at 37°C). Guengerich, Geiger et al. (1981) found (after a 40 h incubation at 37°C in phosphate buffer) that CNEO formed an adduct with the base adenosine. From a TLC plate, the adduct appeared to be 1,N^6-ethenoadenosine. Cytidine and CNEO, under the same conditions, formed an unknown adduct (that was not the expected 3,N^4-ethenocytidine).

9.3.3 *In Vitro* Nucleoside Studies

In the early work by Ofengand (1967), RNA nucleosides were incubated with AN, and the rates of reaction and types of adduct were determined. Adducts to pseudouridine, inosine, thiouridine, ribothymidine, adenosine, guanosine, and cytidine were recognized. These were mostly either carboxyethyl or cyanoethyl adducts to the bases themselves. However, in several instances, the adduct was to the ribose portion of the nucleoside. These were non-enzymatic reactions, carried out at 30°C for 2 to 3 h.

Solomon et al. (1984), and Solomon and Segal (1985) also studied the non-enzymatic addition of AN to 2'-deoxyribonucleosides. They found adducts (after 40-d incubations) formed for all four deoxynucleosides (Table 9.2).

9.3.4 *In Vitro* Studies with Polynucleotides

Guengerich, Geiger et al. (1981) published experiments in which poly-adenylic acid (poly A), polycytidylic acid (poly-C), polyguanylic acid (poly-G), and polyuridylic acid (poly-U) were reacted with AN in the presence of rat liver microsomes and a NADPH regenerating system.

They found some unstable binding of labelled AN to each of these but did not attempt to identify the adducts. Peter, Appel et al. 1983 also

Table 9.2. Adduct derivatives formed from acrylonitrile *in vitro*[a]

Adduct Derivatives	Abbreviations	Percentage[b]
1-(2-Carboxyethyl)-deoxyadenosine	(1-CE-dAdo)	(26%)
N^6-carboxyethyl-deoxyadenosine	(N^6-CE-dAdo)	(8%)
3-Carboxyethyl-deoxycytidine	(3-CE-dCyd)	(1%)
7-(2-Cyanoethyl)-guanine	(7-CNE-gua)	(26%)
7,9-bis-(2-Cyanoethyl)guanine	(7,9-bis-CNE-Gua)	(4%)
Imidazole ring-opened 7,9-bis-(2-cyanoxyethyl)-guanine	(IRO-7,9-bis-CNE-Gua)	(19%)[c]
3-(2-Cyanoethyl)-deoxythymidine	(3-CNE-dThd)	(16%)
1-(2-Cyanoethyl)-deoxyinosine	(1-CNE-dIno)	

[a]Acrylonitrile was incubated 40 d at 37°C at neutral pH with DNA or nucleoside. No concentration was given for DNA but 1 M AN was used for the deoxynucleoside; this is assumed to be the same for DNA (Solomon and Segal 1985).
[b]Percentage distribution in DNA.
[c]Formed after depurination of 7-CNE-GUA from DNA.

observed some binding of labelled AN to the four homopolymers in the presence of a rat liver microsome system. Upon passing the reaction products through a hydroxyapatite column, the radioactivity greatly decreased. No radioactive nucleotides were recovered after hydrolysis of poly-A and poly-C.

9.3.5 In Vitro DNA Studies

Four different laboratories have looked at the capability of AN to produce DNA adducts *in vitro*. Guengerich, Geiger et al. (1981) found some labelled DNA when calf thymus DNA (CT DNA) was incubated in the presence of AN and a rat liver microsome preparation. They also found

labelled RNA when yeast RNA was used in similar fashion. In later work, Hogy and Guengerich (1986) found N^7-(2-oxoethyl)deoxyguanine when CT DNA was reacted with the AN metabolite, CNEO, in the absence of any enzymatic system. Solomon and Segal (1985) and Solomon et al. (1984) found cyanoethyl and carboxyethyl deoxyadenosine, cytosine, thymine, and guanosine adducts when AN was reacted for 40 days with CT DNA in a reaction that included no enzyme system.

Peter, Appel et al. (1983) found that CT DNA had adducts after non-enzymatic reactions ranging from 0 to 100 h with AN. The amount of bound radioactivity decreased after hydroxyapatite column chromatography. In studying the enzymatic binding of AN to DNA, they found that the presence of a rat liver microsome system did permit some binding of labelled AN to the DNA.

In a combination of *in vitro* and *in vivo* studies, Geiger et al. (1983) incubated rat hepatocytes with AN and included CT DNA in the system supernatant. The question of whether or not reactive intermediates could react with extracellular DNA was asked with the isolation of both the CT DNA and the rat hepatocyte DNA. They found a very low level of labelled bases in the CT DNA and none in the hepatocyte DNA.

9.3.6 DNA Adducts in Common with Other Chemicals

AN forms several DNA adducts that are also produced by other chemicals. These DNA adducts are: 1-(2-carboxyethyl)deoxyadenosine, N^6-carboxyethyl deoxyadenosine, and 3-carboxyethyl deoxycytidine. These adducts are formed by a Dimroth rearrangement (Segal, Maté, and Solomon 1979). β-propiolactone and acrylamide have also been found to form these adducts when incubated *in vitro* with calf thymus DNA (Segal, Maté, and Solomon 1979, Segal et al. 1981; Solomon et al. 1984, 1985).

9.3.7 Binding to Proteins

Peter and Bolt (1981) found that rat microsomal binding of AN was increased after the enzymes had been heat inactivated. This non-enzyme-mediated binding of AN to protein is probably the result of alkylation of protein cysteinyl-SH groups of proteins (Geiger et al. 1983). Another part of the Peter and Bolt study, using radioactive AN injected either i.p. or i.v. into rats showed the highest binding of label to liver and spleen, and then lungs, kidneys, and small intestine. The brain was not one of the sites of highest binding, although brain and other CNS tumors are found in rats exposed to AN.

9.4 DNA DAMAGE STUDIES

In a study comparing the ability of AN and CNEO to cause strand breakage of SV40 DNA, Peter, Schwarz et al. (1983) found that the parent compound, AN, was unable to cause strand breakages (giving approximately 0.2 strand breaks per strand SV40 DNA). However, after incubation for

17 h with the SV40 DNA at 37°C in pH 7.5 Tris buffer, 1mM, CNEO did cause from one to two strand breaks per strand of SV40 DNA.

Incubation of intact cells with AN gave rise to significant strand breakage. Parent and Casto (1979) found that doses of 200 and 400 μg AN per ml, incubated for 18 h in tissue culture media, were able to cause strand breakage of hamster embryo cell DNA. An *in vivo* study by Hogy and Guengerich (1986) looked for DNA synthesis in rats that were treated with a sublethal dose of AN (50 mg/kg). They found that unscheduled DNA synthesis (determined by the incorporation of ^{3}H-TdR) occurred in rat liver but not in the brain. They also found that normal DNA synthesis in rats treated with AN decreased in the rat brain with no change in the liver.

9.5 *IN VITRO* TRANSFORMATION

Transformation of Syrian Golden hamster embryo cells was observed by Parent and Casto (1979). Cells that were treated for 6 d with AN were morphologically transformed. In addition, treatment with AN produced an increase in virally-caused transformation foci.

Later work by Banerjee and Segal (1986) found that two different types of mouse cell lines could be transformed morphologically by AN. In C3H/10T½ cells, they were able to show morphological transformation and anchorage-independent growth after 48 h AN treatment in tissue culture medium. They also found that NIH/3T3 cells could be transformed and could show anchorage-independent growth.

10. BENZYL CHLORIDE

Industrial uses for benzyl chloride (CAS # 100-44-7) (BC) include dye manufacturing, insecticides, gasoline additives, pharmaceuticals (such as phenobarbital and benzathine penicillin G), synthetic tannins, and benzyl compounds such as benzyl alcohol and quarternary ammonium chloride (Skowronski and Abdel-Rahman 1986, Bunner and Creasia 1982). A major use is the production of butyl benzyl phthalate, a plasticizer for artificial resins (IARC 1976a). The permitted exposure level in the work environment in the U.S. is an 8-h TWA of 1 ppm (≈5 mg/m^3); NIOSH recommends a 15-min ceiling of 1 ppm (NIOSH 1985, ACGIH 1986c). Acute toxic effects include local irritation of the skin, eyes, and respiratory tract and extend to CNS depression and death. Benzyl chloride is weakly mutagenic without, and slightly more mutagenic with, S9 metabolic activation in the Ames *Salmonella typhimurium* strain TA100 and the *E. coli* strain WP2uvrA(pKM101) (Venitt 1982). One study found no evidence for teratogenicity in Sprague-Dawley rats (Skowronski and Abdel-Rahman 1986). In the United Kingdom Environmental Mutagen Society trials, BC was found to be negative for tumor induction by skin painting but did induce some chromosomal aberrations (Parry et al. 1982). Subutaneous injection of BC has produced local sarcomas in rats, while i.p. injection in mice gave no evidence for carcinogenicity (IARC 1976a).

10.1 CHEMISTRY

Benzyl chloride is a colorless liquid with a boiling point of 179.3°C and a freezing point of -39°C. It is not miscible with H_2O but is miscible with ethanol, ether, and chloroform (IARC 1976a, Ashby et al. 1982).

10.2 ABSORPTION

Studies by Stekol (1938 and 1939) indicate that BC is absorbed through the lungs and gastrointestinal tract. Inhalation studies in rats (Skowronski and Abdel-Rahman 1986) showed that blood concentrations reached a steady-state level between the 4th and 6th h of exposure when exposed for 6 h at 20 ppm. The absorption half-life was 0.53 h, and the elimination half life was 1.12 h. They also found that the peak plasma level of a single dose of BC (at 200 mg/kg i.v. in rats) was reached at 15 min with an absorption half life of 2.8 min. Two phases of elimination from the plasma followed: a rapid phase, with a half-life of 38.5 min, and a slower phase, with a half-life of 33.2 h.

Bunner and Creasia (1982) found that a single oral dose of 250 mg/kg or 4 doses of 125 mg/kg killed all female rats, while the males could tolerate up to 6 to 9 doses of 125 mg/kg or 5 doses of 250 mg/kg. In the high dose animals, severe congestion and edema of the heart,

lungs, and liver were the causes of death for the females, while acute myocardial necrosis caused the male rats to die. For the 125 mg/kg dosed animals, severe, acute gastritis and ulcerative squamous stomach were the probable causes of death. Female rats consistently maintained higher blood levels of the ^{14}C-labeled compound than the males did.

10.3 METABOLISM

Dogs, rats, and rabbits excrete BC as N-acetyl-S-benzyl-cysteine (mercapturic acid) (Stekol 1938, 1939). The putative pathway is through a primary reaction with tissue proteins, followed by hydrolysis and acetylation to a mercapturic acid or by further conjugation with GSH.

Bunner and Creasia (1982) used a 25 mg/kg dose containing methylene-^{14}C-benzyl chloride (administered by gastric intubation) to study tissue distribution and excretion in male and female Fischer 344/N rats. They found that at 3, 6, and 24 h after dosing, the level of radioactivity was highest in the stomach and small intestine contents (a mean of 6 to 8% for the two sexes). Next came kidney, liver, blood, lung, heart, pancreas, fat, and right femur muscle tissue. Excretion of 65-75% of the isotope occurred by 24 h.

10.4 DNA ADDUCT FORMATION

10.4.1 *In Vitro* Studies with Bases

Hemminki, Falck, and Linnainmaa (1983) determined that 10 mM guanosine in a pH 7.2 phosphate buffer at 37°C (with 1% dimethyl formamide) and 10 mM BC would react nonenzymatically. They found some fluorescence (excitation spectra of 220-360 nm, emission at 400nm) but did not identify the product.

Moschel, Hudgins, and Dipple (1979) studied the effect that different solvents have on the non-enzymatic benzylation of adenosine and guanosine. Through column chromatography and liquid scintillation counting of the titrated products, they could determine a conversion of 0.01% of the nucleoside to a benzylated product. All reactions were at pH 6.8 to 7.4, at 25°C, for approximately 5 reaction half-times. They found that increasing the dimethylformamide concentration in the mixture (from 1% to 40%) changed the specificity of the benzyl chloride-adenosine reaction. This occurred at concentrations of 3.4×10^{-3} M BC and 1.1×10^{-6} M adenosine. The N^6 position was benzylated to the extent of 0.15% in 1% dimethylformamide, but only 0.02% at 40% dimethylformamide, while the N^1 position was benzylated 0.08% in 1% dimethylformamide and 0.13% in 40% dimethylformamide. A change from 20 to 40% ethanol did not cause as large an effect. For benzylation of guanosine (1.7×10^{-2} M BC and 5×10^{-7} M guanosine), the only concentration of dimethylformamide shown yielded 0.05% benzylation at the O^6 position, 0.33% benzylation at the N^2 position, and 0.72% benzylation at the N^7 position. Increasing the ethanol concentration

from 20% to 40% raised the percentage of N^7 benzylation (from 1.4% to 2.1%), lowered the N^2 benzylation (0.22% to 0.12%), and had no real effect on the O^6 benzylation (0.05% to 0.04%). If the nucleophilicity of ethanol and water are similar, the conditions assumed to be present in a cell would yield results similar to the higher ethanol and water percentage results. However, local conditions do vary. The results above indicate that at least 5 DNA adducts are possible; all are benzylations (i.e., all are $C_6H_5CH_2$-) at four N sites on guanosine and adenosine (N^6 and N^1 for adenosine and N^2 and N^7 for guanosine) and at O^6 for guanosine.

10.4.2 Observed Binding of Benzyl Chloride to DNA and Hb

In order to determine the rate constant for the reaction of BC with guanine-N^7 in DNA, Solveig Walles (1981) incubated labelled BC with mouse spleen cells in pH 7.2 phosphate buffer for 10 to 20 min. The DNA was isolated from the spleen cells. The second-order rate constant for DNA-guanine N^7 alkylation in DNA was found to be 3.3×10^{-4} $L \cdot (g\ DNA)^{-1} \cdot h^{-1}$, assuming homogeneous distribution of the dose in the animal body.

Solveig Walles then injected between 57 and 348 μg of [^{14}C]-BC into the tail veins of mice and determined the radioactivity of the liver, spleen, lung, testis, and brain DNA. The DNA was isolated by precipitation, extraction in chloroform-isoamyl alcohol, and RNAase treatment, and was then hydrolyzed in 1 M HCl at 100°C for 20 min. N^7-(7-benzyl)guanine was used as the known marker. He found that brain and testis DNA had the highest alkylation level (when mice were sacrificed 1 h post injection), then the liver and lung. Spleen DNA had the lowest degree of alkylation. The values shown in the paper are amount of label per individual animal, and there was more than a tenfold difference between the animals in each group. By 24 h after injection, the brain had the greatest decrease in label, followed by testis and liver. The label concentrations in the lung and spleen did not increase. A second experiment showed the same pattern. The conclusion was that the stability of the N^7-(7-benzyl)guanine varies with the organ, possibly due to enzymatic hydrolysis. No other possible adducts were considered.

At the same time, Solveig Walles isolated and precipitated hemoglobin from the injected mice. The rate constant for benzylated hemoglobin was determined by incubation of erythrocytes (at 37°C, phosphate buffer of pH 7.4) with 1.2×10^{-5} M labelled BC. It was shown to be 1.6×10^{-3} $L \cdot (g\ Hb)^{-1} \cdot h^{-1}$. The half-life for BC, calculated from the hemoglobin data, was approximately 1 min. Between 1 and 6 h post injection, there was no increase in the degree of hemoglobin alkylation, but by 24 h after injection, there was an increase. There was no overall correlation found for the amount of hemoglobin alkylation and the alkylation values for the different tissue DNAs. No correlation was found for the changes in degree of alkylation in the tissues with time.

10.4.3 Similar Adduct Formation by Other Chemicals

Moschel, Hudgins, and, Dipple (1979) showed that BC, benzyl bromide, and benzyl tosylate would all react with nucleosides. The same benzyl-nucleoside derivatives were formed using adenosine or guanosine, but the proportion differed with each benzyl halide. The yield of ring-substituted product was greater for Br than Cl and greater for Cl than the tosylate group. It must also be noted that any other chemical which can form a benzyl-DNA adduct could not be distinguished from the adducts formed with BC.

11. ANALYTICAL METHODS

11.1 INDIVIDUAL CHEMICALS

The specific analytical methods used in studying the individual chemicals are summarized in this section.

11.1.1 Ethylene Oxide

Radiometric methods using ethylene-[1,2-^{3}H]-oxide were utilized to measure the degree of alkylation of hemoglobin (Osterman-Golkar 1976). Direct measurement of EO using GC in alveolar air and blood was performed to estimate EO exposure to workers employed at a hospital sterilizer (Brugnone et al. 1986). GCMS was also used to measure hemoglobin adducts of EO in cigarette smokers (Tornqvist et al. 1986).

11.1.2 Styrene

Several spectroscopic techniques were used to identify reaction products of styrene oxide with guanosine (Hemminki and Hesso 1984). These techniques included exchange chromatography, HPLC, UV absorption and MS. About 90% of the total products were identified, of which 57%, 28%, and 15% were assigned to N^7, N^2, and O^6 adducts.

A radiometric technique was used to determine DNA binding of [^{14}C]-styrene in isolated rat hepatocytes (Legraverend et al. 1984).

Reaction products between styrene oxide and deoxynucleosides and DNA were characterized using HPLC, UV absorption, and chemical ionization mass spectrometry (CIMS) (Savela et al. 1986).

A simple fluorescence assay was developed and evaluated to measure alkylation of guanine. The assay was tested for styrene oxide (Hemminki 1979).

11.1.3 Vinyl Chloride

Reverse-phase HPLC was used to detect DNA adducts formed by VC (Barbin et al. 1985). The detection used UV absorption spectroscopy monitored at 254 nm (Barbin et al. 1985, Barbin et al. 1975).

Many investigators used radiometric techniques to study evidence of chloroethylene oxide (CEO) as a reactive metabolite of VC binding to DNA (Guengerich et al. 1981).

Various spectroscopic techniques including UV absorption, fluorescence, infrared, proton nuclear magnetic resonance (NMR), and field desorption (FD) mass spectroscopy were used to characterize modification of deoxyguanosine by CEO (Scherer et al. 1981). The reaction of deoxyguanosine in glacial acetic acid with CEO led to a strong fluorescent product (Scherer et al. 1981). GCMS was used to identify adducts of CEO with pyrimidine nucleosides (Barbin et al. 1986).

Fourier Transform NMR and GCMS were used to characterize cytosine adducts of CEO (O'Neill et al. 1986).

The reaction of VC with DNA can form etheno products that may play an important role in carcinogenicity. In contrast to unmodified bases and nucleosides, which generally do not fluoresce, two known adducts, 3,N^4-etheno-deoxycytidine and 1,N^6-etheno-deoxyadenosine, are fluorescent and can be detected by fluorimetric techniques (Barrio et al. 1972). More recently, N^2,3-etheno-deoxyguanosine (2,3-EG) has been detected using a fluorescence HPLC method (Oesch and Doeriger 1982). Recently, the quantitation of etheno adducts by fluorescence detection was evaluated (Bedell et al. 1986). Fluorescence sensitivity and selectivity were evaluated using three HPLC systems for two etheno adducts. Enzymatically digested DNA that had been incubated with chloroacetaldehyde was analyzed in the study (Bedell et al. 1986). The method appears to be most sensitive for the ethenodeoxyadenosine adduct, with a limit of optical detection (LOD) of 1 pmole per injection in the HPLC instrument. This sensitivity level is similar to that for O^6-methylguanine, another promutagenic DNA adduct analyzed by HPLC and fluorescence detection.

11.1.4 Epichlorohydrin

Hemminki has tested a simple fluorescence assay for ECH (Hemminki 1979). The assay was devised to measure alkylation of guanine. The reaction products with substrates were analyzed by thin-layer chromatography (TLC). The binding assay measures the change in fluorescence of DNA bases upon covalent binding.

Epichlorohydrin was reacted with deoxyguanosine, and the reaction mixtures were analyzed using HPLC and UV absorption spectroscopy (Hemminki and Lax 1986). HPLC and UV absorption techniques were also used to analyze the reactivity of ECH with deoxynucleosides and DNA (Djuric et al. 1986).

11.1.5 Propylene Oxide

The reaction products of PO with deoxynucleosides and DNA were analyzed using HPLC, UV, and mass spectroscopies (Djuric and Sinsheimer 1984, Djuric et al. 1986). A simple assay, based on the change of fluorescence of DNA bases upon covalent binding with simple epoxides, has been developed and tested for PO (Hemminki 1979).

11.1.6 MOCA

Current techniques for monitoring MOCA levels in urine are summarized in Ward et al. (1986). TLC is semiquantitative, using a visual comparison of the color derived with the standards. NIOSH recommends the use of electron capture GC. This technique is quantitative with detection limits below 1 μg/L urine. HLPC is the third common technique. However, in Ward et al. (1986), results on the same samples evaluated by different testing laboratories and by

different techniques did not agree with each other, or with the known concentrations. Variations in technique and sample storage differences undoubtedly contributed to these variations. For instance, the urine dilution concentration was not taken into account uniformly. Also, glucuronide and sulfate conjugates could not be measured unless they were first acid hydrolyzed. A study by Linch et al. (1971) also found little correlation between GC and TLC results. The TLC technique, described by Ducos, Maire, and Gaudin (1985), involved extraction of the urine by a modified Extrelut cartridge (Merck) and then methylene chloride extraction. The HPLC was performed using a Partisil 10 PAC cyanoamino column. Still another technique (Van Roosmalen, Klein, and Drummond 1979) used partial purification of MOCA by solvent extraction and TLC prior to the GC step.

11.1.7 Benzidine Dyes and Benzidine

Josephy and Iwaniw investigated the reaction of BZ with N-acetylcysteine (Josephy and Iwaniw 1985). The adducts were separated by HPLC and characterized by UV absorption using a diode array spectrometer equipped with a flow cell. The major product was isolated, analyzed by NMR, and identified as 3-(N-acetylcystein-S-yl)-benzidine.

The binding of BZ diimine with DNA was characterized using HPLC, UV, and NMR (Yamazoe et al. 1986).

A radiometric technique using [^{14}C]-BZ was utilized to study DNA binding (Tsuruta, Josephy et al. 1985).

11.1.8 Acrylonitrile

Binding of AN to nucleic acids was generally measured by using radiolabeled compounds such as [2,3-^{14}C]-AN (Peter et al. 1983). Farooqui and Ahmed used radiolabelled [2,3-^{14}C]-AN to study binding to protein, RNA and DNA (Farooqui and Ahmed, 1983).

UV spectroscopy, electron impact (EI), desorption chemical ionization mass spectroscopy (CIMS) and Californium-252 fission fragment ionization MS were used to study direct alkylation of AN to DNA *in vitro* (Solomon and Segal 1985, Solomon et al. 1984).

Hogy and Guengerich used several techniques to investigate the carcinogenic mechanism of AN and its epoxide (CNEO) in rats (Hogy and Guengerich 1986). Radioactive 2-cyano [2,3-^{14}C]-ethylene oxide was administered to rats i.p., and radiometric derivative assays could detect low levels of N^7-(2-oxoethyl)guanine at 0.014-0.032 alkylations per 10^6 DNA bases.

HPLC-fluorescence and TLC methods were also used to detect other types of adducts such as 1,N^6-ethenoadenosine and 1,N^6-ethenodeoxyadenosine (Hogy and Guengerich 1986).

11.1.9 Benzyl Chloride

Radiometric techniques using [^{14}C]-BC were used to investigate toxicity, tissue distribution, and excretion of BC in the rat (Bunner

and Creasia, 1982). The effects of orally administered BC on fetal development were also evaluated in laboratory rats (Skowronski and Abdel-Rahman,1986). Studies were conducted to evaluate BC-induced DNA damage and to classify the strand breaks detectable directly in BC-treated cells (Mirzayans and Waters, 1985).

11.2 GENERAL DISCUSSION ON ANALYTICAL METHODS

In most studies of the chemicals of interest, DNA interactions have been investigated using radiometric techniques. The interactions of chemicals with DNA were measured both at a gross level, i.e., total binding of compounds, and at the molecular level, i.e., reactions with specific DNA products. Radiometric techniques generally suffer from several disadvantages: radioisotopes have limited shelf life (60-d half-life for ^{125}I); instruments for detecting and reagents are relatively expensive; and special precautions are required for the shipping, handling, and especially the waste disposal of radioactive materials. Furthermore, high sensitivity requires using high specific activity isotopes. Potential confounding biological effects due to the radioactive labels are also of concern. The limits of radiometric detection for the compounds reviewed here are in the range of 1 adduct per 10^7 or 10^8 base pairs in 50 μg DNA.

Several techniques that do not require radioactive labels were used to determine the amount of alkylation in nucleic acids and proteins. Chromatographic methods such as HPLC GC/MS have been applied to measure adduct formation from alkylating agents, such as methylating and hydroxyethylating compounds, reacting with amino acids in proteins. In general, the GC/MS techniques are more elaborate and more time-consuming than the immunological methods developed to detect DNA adducts.

NMR has also been used to investigate DNA alkylation products (Hemminki et al. 1980a,b). The NMR technique is generally useful for structure determination but is not practical for routine analysis since it requires sophisticated instrumentation and skilled workers, and has limited sensitivity.

Although techniques such as ^{32}P-labeling and radioimmunoassay (RIA) methods have achieved excellent sensitivities with chemicals other than those of interest here, alternative techniques are required to overcome the need for radioactive labels. Recent progress in advanced spectroscopic techniques, sensor development, and biotechnology have opened new possibilites for the development of sensitive and selective tools to measure DNA adducts.

As discussed previously, a fluorescence technique has been used for a variety of epoxides related to propylene oxide, glycidol, epichlorohydrin, trichloropropylene oxide, and styrene oxide (Hemminki 1979). The sensitivity and selectivity of this method can be further improved by developing the synchronous luminescence (SL) technique. The SL technique (Vo-Dinh 1978; Vo-Dinh 1982) has been used to detect benzo(a)pyrene-diol-epoxide (BPDE) adducts (Vahakangas et al. 1985). BPDE is the ultimate carcinogenic metabolite of benzo(a)pyrene (BP), a

polynuclear aromatic hydrocarbon (PAH or PNA). The technique can be further developed to measure fluorescent adducts of chemicals of interest. The SL approach could provide a cost-effective and practical tool to monitor human exposure to genotoxic chemicals.

Recently, room temperature phosphorimetry (RTP) was used to detect BPDE-DNA adducts (Vo-Dinh and Uziel 1987). The RTP of benzo(a)pyrene-r-7, t-8,9,10-tetrahydrotetrol (BP-tetrol, BPT) was measured using laser excitation. The BPT was obtained by acid hydrolysis of BPDE-DNA adducts. The BPT sample was measured on a filter paper substrate pretreated with a heavy-atom salt, thallium acetate, which was used to increase the phosphorescence signal of BPT. The detection limit of BPDE in *in vitro* modified BPDE-DNA was about 15 femtomoles (10^{-15} moles). Although the spectral selectivity of RTP spectra is less than that of high-resolution low-temperature spectra, the method is characterized by its simplicity and does not require sophisticated and expensive instrumentation or cryogenic equipment and refrigerant. Thus it is well suited for routine analysis and screening procedures. Other advantages of this technique are the absence of extensive cleanup procedures and a reasonably good sensitivity. The RTP approach could provide an additional practical tool to monitor human exposure to carcinogenic and mutagenic compounds of interest in epidemiological studies.

Another novel technique of great promise is the surface-enhanced Raman scattering (SERS) technique. The application of Raman spectroscopy for the study of biological systems is rapidly expanding because of the specificity of this analytical technique for chemical identification. Conventional Raman spectroscopy, however, has limited applicability for trace organic detection because of the inherently weak Raman scattering cross section. The SERS effect (Chang and Furtak 1982), whereby an enhancement factor of up to 10^6 in Raman signals from molecules adsorbed on rough metallic surfaces is obtained, has recently generated increasing interest in the Raman technique. The technique can be used to analyze compounds of biological interest such as BPDE-DNA adducts, DNA, and BPT. Especially for biological samples, conventional Raman spectroscopy has the disadvantage of requiring large samples (usually 10-100 mg of the bulk pure specimens). The increased sensitivity provided by the SERS effect has eliminated this major limitation. BPDE-DNA adducts were analyzed recently by SERS (Vo-Dinh, Uziel, and Morrison 1987). The general applicability of SERS was extended by the use of a practical and easy-to-prepare substrate. The production of metal surfaces with submicron roughness is a prerequisite for inducing the SERS effect in adsorbed molecules. The technique for preparing the silver-coated cellulose-based substrates in this study is simple and efficient in producing SERS-active substrates. In this study, samples of only 5 μL were required. In this small sample, the amount of BPT sample analyzed was only 30 picomoles. The study indicates that the adduct can be identified in the DNA sample without requiring chromatographic separation of the BPT species from the DNA products. The SERS technique appears to have great potential for other DNA-adduct systems.

For the past few years, biosensor technology has been at the forefront of analytical instrumentation research. The integration of biological systems and advanced optical sensor technology promises to open new horizons in environmental and biological monitoring applications. Recently, a fiberoptics antibody-based fluoroimmunosensor (FIS) was developed for BP (Vo-Dinh, Trombert, Griffin, Ambrose, et al. 1987). Polyclonal antibodies produced against BP were immobilized at the terminus of a fiberoptics probe within the FIS for use in both *in vitro* and *in vivo* fluorescence assays. High sensitivity was provided by laser excitation and optical detection. The excellent sensitivity of this device (1 femtomole limit of detection for BP in a 5 μL sample drop, [Vo-Dinh, Tromberg, Griffin, Ambrose, et al. 1987]) illustrates that it has considerable potential to perform trace analyses of chemical and biological samples in complex matrices. Measurements are simple and rapid ($\approx$12 min), and the technique is applicable to other compounds provided appropriate antibodies are used. For example, an FIS device for BPDE-DNA adducts (actually for BPT hydrolyzed from the BPDE-DNA adducts) has recently been developed using monoclonal antibodies (Vo-Dinh, Tromberg, Griffin, Sepaniak, et al. 1987). The BPT has been detected in the subfemtomole range. The FIS technology can be further developed and extended to the detection of other chemicals of interest.

12. RESEARCH NEEDS

The idea that measurement of cellular molecular events will provide the basis for descriptive toxicological theory is not new. The application of these approaches to making epidemiology a more rigorous science is also not new. The design of a rigorous molecular epidemiological study for the chemicals in this review, which will contribute toward an improved method for risk analysis, will require more advanced methodologies than are now available. The specific research needs that have been identified are as follows.

* **Sensitive and practical detection methods are needed for all the studied chemicals.** At this time, a goal of detecting at least one adduct per 10^9 base-pairs of DNA in a tissue sample containing less than 50 μg of DNA (less than 0.2 femtomole per 50 μg DNA) seems attainable. This is about half the amount of DNA expected in a 40 ml blood sample. If, however, initiation of latent disease can be produced by low-level exposures resulting in only a very few adducts (e.g., initiating a single cell), then a much lower level of detection will be required. In addition, the technique should give some qualitative information about the structure of the adduct in order to assure that the adduct is derived from the chemical of interest. This combination of high sensitivity and qualitative identification is needed to establish that minor adducts, with significant genotoxic potential and perhaps greater potential for estimation of risk, are not overlooked.

* **Criteria for selection of the tissue to be monitored should be developed that determine whether or not the disease target tissue needs to be monitored, and whether or not there are satisfactory surrogate tissues that could be used as the adduct source.** The lifetime or turnover rate of the adduct in the tissue should be known, along with the level of a stable adduct within the tissue, so that an estimate of the initial level of adduct formation as well as the persistent level of formed adduct can be made. The stable adduct does not need to be the critical DNA adduct. These types of data would help to reduce uncertainty in the subsequent estimation of risk.

* **Two types of sampling problems need to be addressed.** First, calculations need to be done to determine the number and type of measurements that will be required to obtain significant measures of exposure that relate to the endpoint of interest. Second, specific procedures must be worked out for the obtaining and handling of samples for each chemical that include protocols for taking blood or other tissue samples, fractionation or processing if necessary, storage, and transport to the laboratory for adduct analysis.

* **There is a need for adduct selection criteria to ensure that the DNA adduct to be measured has either, or both, a causal link to genotoxicity or other specific measures of latent disease, or is directly linked to internal exposure.** An as example of the potential complexity of adduct selection, 7-alkyl-guanine adducts have been characterized as both nongenotoxic and genotoxic depending upon the 7-

alkyl substituent. In addition, there is the need to select unique DNA adducts to minimize uncertainty from possible multiple chemical exposure sources of the adduct. Table 12.1 shows groups of chemicals that give rise to the same adduct(s).

Table 12.1. Potential concomitant exposures in otherwise suitable population group

Chemical	Concomitant exposure
Ethylene oxide	Antineoplastic drugs
Styrene	Acetone Methylethylketone
Vinyl chloride	Ethylene Dialkylphthalates
Epichlorohydrin	Cis-trans isomers of 1,3-dichloropropene 1,2,3-Trichloropropane
Acrylonitrile	Styrene Butadiene Dimethylformamide Thiocyanate Azoisobutyronitrile
Benzyl chloride	Toluene

As an alternative to monitoring unique DNA adducts, measurements of specific batteries of adducts and/or other molecular responses might provide measures of unique exposures because of the low probability of a spontaneous observation of several unrelated events. The theory of sampling frequency needs to be fully defined and implemented for optimal collection of data.

Furthermore, the population to be studied should be selected primarily for a history of exposure exclusively to the chemical of interest. Table 12.2 shows other chemicals that subsets of the potential study population may also have been exposed to, and that could have confounding effects on the results of the study.

By limiting the population to a single exposure group and selecting a unique DNA adduct, the theoretical concepts of molecular epidemiology can be relatively easily monitored. Furthermore, the idea that sources of variability can be confined to the variations in exposure and to the interindividual differences in extent of adduct formation per unit dose of chemical can be tested.

Table 12.2. Chemicals that give rise to the same adduct(s)

Adduct	Chemical
N^7-(2-HOEt)-guanine[a]	Ethylene oxide N-nitrosomethylethylamine N-nitrosodiethylamine
N^7-(2-oxoethyl)-guanine[a]	Vinyl chloride Acrylonitrile 1,2-Dichloroethane 1,2-Dibromoethane Ethylene halohydrins Urethane Glycol --> glycolaldehyde Ethylene --> ethylene oxide --> glycolaldehyde
N^7-methylguanine	*In vivo* hydrazine plus endogenous formaldehyde *In vivo* formaldehyde plus hydrazine Methylnitrosourea Methylnitrosamine Dimethylnitrosamine Dimethyl sulfate Methyl methane sulfonate
O^6-methylguanine	*In vivo* hydrazine plus endogenous formaldehyde *In vivo* formaldehyde plus hydrazine Methylnitrosourea Methylnitrosamine Dimethylnitrosamine Dimethyl sulfate Methyl methane sulfonate
Carboxyethyl-substituted bases	Acrylonitrile β-Propiolactone Acrylamide

[a]Adducts to other bases may also be formed.

* **There is a need to develop mathematical tools for using DNA adduct level information in a predictive mode.** This implies development of non-statistical means of describing the relationship between adduct and exposure, or adduct and observed endpoint, or adduct and latent

disease. As part of this research need, there is a need to develop an understanding of how exposures that are irregular in time and variable in intensity and duration can be accurately quantified by measurements of molecular indicators of internal exposure. The definition of general--and rigorous--self-consistent units for describing exposure and genotoxic effects is needed. Rate measurements should be clearly distinguished from absolute quantities. The units must be useful for transpecies calculations.

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APPENDIX A
ADDUCT FORMATION AND ESTIMATION OF DOSE, EXPOSURE MONITORING, AND RISK ESTIMATION

A.1 ESTIMATION OF DOSE FROM DNA OR Hb ALKYLATION

Ehrenberg et al. (1974) proposed the idea that the proportion of adduct formation among different macromolecules was primarily dependent on the concentration of reactive chemical species in the body. Assuming a uniform distribution of the reactive chemical and stability of the adduct formed, the degree of alkylation is directly proportional to the dose according to the following equation:

$$[RY]/[Y^-] = k_y \cdot D \qquad (A.1)$$

where k_y is the second order rate constant for the reaction of EO with the macromolecule (Eq. [A.3]), and D is the time integrated dose (A.2) of alkylating agent RX. In Eq. (A.3), RY is the number of adducts and Y^- is the amount of macromolecule such that the ratio is the adduct frequency per g of substance.

$$D = k_y \int_{t_o}^{t} C(t)\, dt \qquad (A.2)$$

$$RX + Y^- \xrightarrow{k_y} RY + X^- \qquad (A.3)$$

The concentration variable C is calculated from the estimated alveolar ventilation (minute volume), time of exposure, and concentration of gas in the experimental atmosphere. The concept is used to estimate DNA adduct formation by assuming a direct proportionality between the second order rate constants of adduct formation and the total amount of product formed. For example, by measuring the amounts of Hb adducts and using the measured rate constants for formation of Hb adducts and DNA adducts, the amount of DNA adduct formed can be estimated:

$$k_{yHb} \,/\, k_{yDNA} = [Hb\text{-}adducts] \,/\, [DNA\text{-}adducts] \qquad (A.4)$$

Since [*Hb-adducts*] is the value for the measured $[RY]/[Y^-]$ for Hb, the result from solving Eq. (A.4) [*DNA-adducts*] can be put into Eq. (A.1) to solve for D, which is the tissue dose of reactive chemical species. When this value is divided by the measured value of k_y, the time-integrated concentration of RX (i.e. the reactive chemical species of a given chemical) can be estimated from Eq. (A.2).

A.2 USE OF THE PROPORTIONALITY RELATIONSHIP TO EVALUATE THE RELATIVE BIOLOGICALLY EFFECTIVE DOSE

Using the concept of target dose proportionality to concentration of reactive species (Ehrenberg 1979), a series of monofunctional alkylating agents were compared at equal doses as mutagenic agents in barley (Osterman-Golkar, Ehrenberg, and Wachtmeister 1970) and microorganisms (Turtoczky and Ehrenberg 1969, Hussain and Ehrenberg 1975). The data were used to make a biological reactivity comparison similar to Swain and Scott's (1953) comparison of alkylation effectiveness and mechanism of action (first order carbonium intermediate compared to second order displacement). The observed "n" values reflect the relative effectiveness of the two mechanisms; reactive oxygen atoms give Swain-Scott n values of 0-4, reactive nitrogens give values of 3 to 5, and compounds with reactive sulfur give values of 5-7.

Ehrenberg (1979) and Ehrenberg and Osterman-Golkar (1977) observed a proportionality between mutation frequency and chemical reaction rate when the "n" value was about 2. The "n" value for N^7-guanine is about 3.5. They suggest that this probably excludes alkylation at guanine N^7 as the source of mutagenic action. The O^6 guanyl position has an "n" value of 1.2 and is considered within the range of values consistent with induction of mutagenesis.

A.3 APPLICATION OF ADDUCT FORMATION TO EXPOSURE MONITORING

Osterman-Golkar et al. (1976) evaluated the use of Hb adduct formation as a dose monitor when using male CBA mice, 6-8 weeks old. They observed a linear decrease in 3-methylhistidine-Hb after a single intraperitoneal injection of dimethylnitrosamine (DMNA) which extrapolated to zero adduct present at about 39 days. DMNA requires metabolism before forming an alkylating agent. They suggest that EO-Hb adducts also follow a linear decay curve and that the shape of the accumulated adduct formation vs repeated dose curve will follow the equation:

$$dy/dt = a - at/t_{er} \quad (t \leq t_{er}) \tag{A.5}$$

They suggest that Hb adducts will be valuable as surrogate monitors of exposure since the accumulated extent of Hb alkylation, with constant exposure, will follow the integrated equation

$$y = a(t-[t^2/2t_{er}]) \tag{A.6}$$

where y is the observed adduct frequency. They also suggest that the level of alkylation y will reach a plateau at time t_{er}, the estimated lifetime of the animal's red cells (Table A.1). On inspection of this equation, it is clear that the slope of the curve dy/dt becomes zero (i.e. constant y), when $t = t_{er}$.

Table A.1. Red cell life-span

Reference	Animal	Life-span
Osterman-Golkar et al. (1983)	Fischer 344 rat	60 days
Segerback 1983	CBA mouse	40 days
Segerback 1983	human	120 days

Use of this concept for DNA adduct formation is more complex because of the different lifetimes of the different leukocyte populations (see Table A.2).

Table A.2. Distribution and DNA content of human leukocytes

Cell[a]	Cells per mL blood[b]	DNA μg/mL blood[c]	Lifetime[d]
Lymphocytes (33%)	2.5×10^6	15	3 yr av[e]
Monocytes (6%)	0.45×10^6	2.7	3-4 d
Neutrophils (59%)	4.42×10^6	26.5	< 1 d
Others (2%)	0.15×10^6	0.9	< 1 d

[a]Cell distribution is given in parentheses (Altman and Dittmer 1961).
[b]Based on observed cell numbers (Altman and Dittmer 1961).
[c]Based on 6 pg of DNA/cell and 100% yield of cells.
[d]Doull, Klaassen, and Amdur 1980.
[e]Preston 1982.

This is the mean half-life of the circulating pool of peripheral lymphocytes with some proportion lasting over 20 years.

A.4 CORRELATION OF EXPOSURE TO RISK

Ehrenberg et al. (1974) have developed an hypothesis that correlates the level of tissue protein binding to the absorbed tissue dose based on the observed apparent second-order kinetics of binding and the rapid

body distribution and excretion of hydrolyzed ethylene oxide. By using the value of 4×10^{-4} (L/g/h) for the second order rate constant and 0.23 nmol hydroxyethyl-amino acid per g protein per ppm·h as the dose-dependent binding rate (based on the average for spleen and testes protein), the following relationship between tissue dose and exposure has been developed:

$$D_t = 0.58\ \mu M \cdot h \text{ per ppm} \cdot h \qquad (A.7)$$

By using the relative pulmonary ventilation rates of man and mouse, and assuming the half-life of ethylene oxide in man is the same as in the mouse, they calculated the corresponding absorbed dose rates for man:

Man at rest: $D_t = 0.06\ \mu mole \cdot kg^{-1} \cdot h/ppm \cdot h$
Man at moderate exercise: $D_t = 0.28\ \mu mole.kg^{-1} \cdot h/ppm \cdot h$

As part of Ehrenberg's hypotheses to correlate chemical and radiation damage risk estimates, they have used the extent of mutation (chromosomal) damage to calculate equivalent doses for radiation in rads and ethylene oxide absorbed dose (D_t). These data were used to support the concept that ethylene oxide is a significant risk to man.

A.5 RISK ESTIMATION FROM LEVELS OF ADDUCTS

Ehrenberg (1974) suggested that agents like EO, which are rapidly distributed throughout the body in their most reactive forms, would demonstrate dose response relationships like radiation exposures and that it may be possible to estimate human risk from chemical exposure by using the corresponding tissue dose levels of radiation and human radiation risk estimates.

Ehrenberg (1980) and Ehrenberg and Osterman-Golkar (1977) used a modified form of the Swain and Scott (1953) relationship describing chemical reactivity of alkylating agents. By comparing the relative rates at a nucleophilicity value of n=2, they observed proportionality:

$$\text{mutation frequency} \approx (RY_{n=2})/(Y^-_{n=2}) \qquad (A.8)$$

This permits calculation of the relative potency of each agent when compared to the reference radiation-induced mutation frequency (The term RY/Y^- is the adduct frequency). Thus an ionizing radiation dose of 1 rad was found to correspond to an adduct frequency of 1×10^{-7}. Ehrenberg (1980) and Ehrenberg and Osterman-Golkar (1979) have developed the following expression for expressing chemical risk in rad-equivalents:

$$\text{risk} = D \cdot k_{n=2} \cdot 1 \times 10^7 \cdot \Pi_i f_i \text{ rad-equivalence.} \qquad (A.9)$$

Using a value of one for Πf and the retention time of 4.6 h^{-1} for EO in mice, a unit risk value for EO to man was estimated to be equivalent to 11 mRad (rounded off to 10 mRad equivalents). This is still a tentative value that was judged to be correct within a factor of two (Ehrenberg and Hussain 1981).

By using dose-effect curves of EO and X-rays in barley, a tissue dose of 1 mM·h may be tentatively set at 80 rad of low LET radiation. An example of epoxide operators working at 5 ppm EO for 40 h/week is estimated to give a weekly gonad dose of EO amounting to 4 "rad-equivalents". Ehrenberg et al. (1977) use these relationships to estimate the risk from exposure to ethene. They indicate that mean ethene concentrations in some American cities may be 50 ppb. They estimate that this represents a genetic risk of about 1 mrad-equivalent per ppm·h. An estimate of risk from ethene in cigarette smoke (20 cig/d) amounted to 30 mrad-equivalents per week.

Tornqvist et al. (1986) have estimated cancer risk from exposure to EO generated from ethene by cigarette smokers in Sweden. They estimate an average dose of 10 cig/d and they calculate the annual dose as 13 x 10^{-6} Mh/year or 1 rad-equivalent per year. This was calculated from the Hb adduct levels and an estimated genotoxic potential of 0.8 Gy whole body gamma radiation from 1 μM·h EO exposure. They estimate an excess cancer risk from smoking of ~4 x 10^{-4}/year or 16% of the estimated cancer risk in the average male smoker in Sweden at ages 18 or older.

APPENDIX A

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