



URINARY THIOETHER EXCRETION AS AN INDEX
OF OCCUPATIONAL CHEMICAL EXPOSURE

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for the degree of Doctor of Philosophy

by

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CONTENTS

	Page
ABSTRACT	i
DECLARATION	iv
ACKNOWLEDGEMENTS	v
ABBREVIATIONS	vi
CHAPTER 1: General Introduction	
1. The Development of Occupational Toxicology	1.1
2. Strategies for Detecting Chemical Hazards in the Workplace	1.2
3. Role of Metabolism in Chemical Toxicology	1.6
4. Role of Glutathione-S-Transferase and Other Detoxifying Enzymes	1.7
5. Urinary Thioether Excretion	1.9
6. Aims of this Thesis	1.15
CHAPTER 2: Factors Modifying Urinary Thioether Excretion in an Animal Model	
Introduction	2.1
Methods	2.4
Results	2.8
Discussion	2.10
CHAPTER 3: Urinary Thioether Elimination by Control and Occupationally-Exposed Populations	
Introduction	3.1
Methods	3.4
Results	3.7
Discussion	3.9
CHAPTER 4: Applications of the Thioether Technique in Assessment of Styrene Exposure of Man and the Rat	
Introduction	4.1
Methods	4.4
Results	4.8
Discussion	4.12
CHAPTER 5: General Discussion	5.1
APPENDICES:	
1. Determination of Urinary Thioether Concentration	
2. Determination of Urinary Creatinine Concentration	
3. Determination of GSH-S-Transferase Activity	
4. Occupational Chemical Study - Questionnaire	
5. Standard Curve for Mandelic Acid	
6. Standard Curve for Phenylglyoxylic Acid	
BIBLIOGRAPHY	

ABSTRACT

The aims of this thesis were to assess the utility of the thioether technique as an index of chemical exposure, and investigate parameters modifying thioether excretion.

Validation of the assay system was performed using the rat as an animal model. Urinary thioether excretion following administration of benzo(α)pyrene, diethyl maleate and o-xylene was shown to be dose-related and sensitive to microsomal monooxygenase induction.

The technique was then applied to a control human population, not exposed to any appreciable chemical levels, and three different occupational groups. Urinary thioether output of control subjects was consistently low and independent of diurnal variations in enzyme activity. Heavy smokers, smoking more than twenty cigarettes per day, tended to eliminate significantly higher levels of thioethers.

Generally, thioether output of all three occupational groups, research laboratory staff, petrol station attendants, and fibreglass workers was elevated following a working day. The magnitude of the increase was indicative of the intensity of exposure to occupational chemicals in the workplace.

Work-related thioether output was enhanced amongst smokers for all three groups studied. For petrol station attendants, an interactive effect between chemical exposure and cigarette smoking on thioether elimination was demonstrated using chi-square contingency analysis. However, due to the variability of employment duties and conditions for fibreglass workers, no significant interactive effect between these two parameters was demonstrated.

Isolation of petrol station attendants from petroleum fumes, as in

self-service outlets, markedly reduced urinary thioether output. Additionally, analysis of the data using the one sample runs test showed that thioether elimination was not influenced by alcohol intake or performance of mechanical workshop duties. Therefore, inhalation of petroleum fumes was implicated as the major source of increased thioether output by driveway attendants.

Initial studies of fibreglass workers involved simultaneous monitoring of excretion of thioethers and specific styrene metabolites, mandelic acid (MA) and phenylglyoxylic acid (PGA), on both working and nonworking days. Significant positive correlations between thioether output and MA, PGA, and combined metabolite excretion implicated styrene as the major source of work-related increased thioether elimination. Animal studies showed that styrene-related excretion was dose-related; however, quantitative interpretations of human data were limited by the lack of information detailing ambient styrene concentrations. Qualitatively, work-related thioether output was exposure-related, as fibreglass laminators, potentially exposed to high styrene levels, excreted markedly higher thioether concentrations than other fibreglass workers.

Evidence from animal studies demonstrated the sensitivity of styrene-related thioether, MA and PGA elimination to enzyme induction, comparable magnitudes of increase in each parameter produced by general inducing treatments. This result tended to confirm enzyme induction produced by cigarette smoking as the main mechanism for smoking-related increases in thioether output.

Pretreatment of rats with beta-naphthoflavone, a cytochrome P-448-specific inducer, produced selective enhancement of styrene-related thioether output. Therefore, toxicology of occupational

chemicals and associated health risks may be influenced by selective induction of different pathways involved in chemical biotransformation.

Follow-up studies of fibreglass workers, in which thioether MA and PGA elimination was monitored over a consecutive eight-day period, revealed no evidence of cumulative effects on thioether excretion. Concentrations of thioethers measured in pre-work samples were consistently low and within the range of levels excreted by a control population. Chronic dosing of rats with styrene, comparable to long-term occupational exposure of fibreglass workers, produced no evidence of auto-induction and hence increased chemical clearance.

The overall results of this thesis indicate that urinary thioether determination is a simple, sensitive, qualitative exposure test, enabling rapid assessments of worker exposure and efficiencies of occupational hygiene practices to be made. Simultaneous monitoring of specific chemical metabolites associated with particular chemical exposures in the workplace can aid interpretation of thioether data.

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge, contains no material previously published by any other person, except where due reference is made in the text.

The experimental work described herein was carried out from 1980 to 1983 in the Department of Clinical and Experimental Pharmacology, University of Adelaide. During this time the author was employed as Tutor in the Department. Funding for technical assistance during August to December, 1981 was provided by the University of Adelaide Foundation.

Results of this thesis have been presented to meetings of the Australasian Society of Clinical and Experimental Pharmacologists in Adelaide (December, 1981) and Melbourne (May, 1982).

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree of Doctor of Philosophy.

Jane Kathryn Stock

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ABBREVIATIONS

GSH	reduced glutathione
-SG	oxidized glutathione
GSHt	glutathione transferase
-SH	sulphydryl
HPLC	high pressure liquid chromatography
SCE	sister chromatid exchange
BZP	benzo(α)pyrene
DEM	diethylmaleate
XYL	o-xylene
PB	phenobarbital
PCB	polychlorinated biphenyl
3-MC	3-methylcholanthrene
BNF	β -naphthoflavone
EDTA	ethylenediamine tetraacetic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic) acid
NAC	N-acetylcysteine
S-9	9,000xg supernatant fraction
CDNB	1, chloro-2,4 dinitrobenzene
MA	mandelic acid
PGA	phenylglyoxylic acid

C H A P T E R O N E

GENERAL INTRODUCTION



CHAPTER 1

General Introduction

1. The Development of Occupational Toxicology

Within the last twenty years significant developments in industry and technology have resulted in a marked increase in synthesis of organic chemicals corresponding with demands for improved living standards. Progress made in the chemical sciences, although benefiting man both economically and socially, also increased hazards associated with exposure to agents potentially noxious to health. Mass exposure, principally within the workplace, intensified with an apparent lack of regard or appreciation of toxicity, carcinogenicity or mutagenicity of chemicals involved.

Various epidemiological studies highlighted associations of occupational chemical exposure and toxicity or carcinogenicity observed (Haley 1975; Heath et al. 1975; Decoufle 1979). Occupational exposure to vinyl chloride monomer, within polyvinyl chloride production operations, was directly linked to an increased incidence of angiosarcoma of the liver, a particularly rare form of liver cancer (Fishbein 1979).

A need to quantitate and regulate chemical exposure within the workplace was generated, thereby enabling minimization of associated health risks. Consequently, within the area of traditional toxicology, primarily concerned with identification of risks to human health (Hodgson & Guthrie 1980), occupational toxicology, defined as the recognition, evaluation and control of chemical exposure and associated health hazard, evolved.

2. Strategies for Detecting Chemical Hazards in the Workplace

Assessment of hazards associated with occupational chemical exposure presents various problems. Exposure of workers to a range of chemicals of differing intensity and frequency may occur, and may be potentially capable of causing additive, antagonistic or synergistic effects, thereby modifying individual chemical toxicology (Bronzetti et al. 1983).

Two main approaches - biological or environmental monitoring - have been developed as a means of estimating health risks associated with occupational chemical exposure.

Environmental monitoring is defined by Berlin et al. (1979) as the systematic collection, analysis and evaluation of environmental samples such as air within the workplace. Exposure of employees of reinforced plastics manufacturing industries has been monitored by measurement of ambient styrene concentrations associated with different working areas in each factory (Götell et al. 1972; Schumacher et al. 1981). Environmental monitoring programmes therefore assess exposure directly, qualitatively and quantitatively.

However, application of environmental monitoring techniques within industry is often inadequate or inappropriate. Biological effects and associated health risks of organic solvents are related more directly to the proportion absorbed by the individual than ambient concentrations within the workplace (Gompertz 1980). Due to large inter-individual variations in solvent uptake in various working conditions, estimations of associated health hazards are more accurately inferred by measuring elimination of chemicals or metabolites in body fluids. Within such industries application of biological monitoring is generally preferred. Biological monitoring is defined by Berlin et

al. (1979) as the systematic collection of human specimens, such as blood, saliva or urine, for analysis of pollutant or metabolite concentrations. Application of this approach within industry eliminates the necessity and relevance of measuring each source of chemical exposure and accounts for differences in chemical pharmacokinetics due to age, sex, weight, health status, inborn errors of metabolism, and genetic or acquired capacity for enzyme induction (Zielhuis 1979). Biological monitoring provides an estimate of internal load or body burden of chemicals more directly related to assessment of potential chemical hazards. Table 1.1 provides examples of this approach currently employed in certain industries.

Prior to the development of a biological monitor or exposure test certain aspects should be considered, namely:

- metabolism of chemicals concerned;
- compound(s) to be measured;
- sample to be analyzed;
- time of sampling;
- storage and preservation of specimens;
- method of analysis;
- expression and interpretation of results

(Bardoděj 1980).

A definite relationship between elimination of compounds assayed in biological specimens and occupational chemical exposure should exist, enabling associations of biological parameter(s) and health effects to be inferred.

The most commonly analyzed biological material is urine (Ikeda 1978). The majority of environmental chemicals undergo biotransformation and clearance via the kidneys, consequently eliminated as polar

TABLE 1.1

Examples of biological monitoring techniques currently in use.

Study	Industry	Chemical Exposure	Metabolites Assayed	Correlation with Ambient Levels	Specimen
van Doorn et al. (1981c)	Chemical, viscose production	Carbon disulphide	2-thiothiazolidine-4-carboxylic acid		Urine
Engström et al. (1978b) Ohtsuji & Ikeda (1970)	Reinforced plastics production	Styrene	Mandelic acid Phenylglyoxylic acid		Urine
Maroni et al. (1981)	Production of electric capacitors	Polychlorinated biphenyls + chlorine	Trichlorobiphenyl Pentachlorobiphenyl Total PCB	0.91 0.84 0.91	Blood
Perbellini et al. (1981)	Shoe manufacture	Commercial hexane (n-hexane) (2-methylpentane) (3-methylpentane)	2,5-hexanedione 2,-hexanol 2 methyl-2-pentanol 3 methyl-2-pentanol	0.67 0.69 0.76 0.65	Urine
Yonemoto & Suzuki	Chemical, polyacrylonitrile and synthetic leather production	Dimethyl formamide	Methylformamide	0.90	Urine

metabolites in urine. Urinalysis of metabolites therefore reflects the amount of chemical(s) absorbed, accounting for differences in work conditions, habits and physical work load. Determination of urinary metabolite concentrations provides a convenient and readily accessible means of monitoring occupational chemical exposure, thereby facilitating worker co-operation and compliance with the programme.

'Spot' collections of urine samples at the end of the working day are generally taken. Time of sampling becomes more critical, however, with assessment of exposure to chemicals with short biological half-lives, sampling required at increased frequency over shorter time intervals. Biological materials collected usually can be stored for at least two weeks at 4°C without degeneration or deterioration of compounds present (Bardoděj 1980).

Analytical procedures should be simple, selective, sensitive, reproducible and rapid, enabling analysis to be performed as often as required. In summary, major determinants of a successful biological monitor include convenience of sample collection, sensitivity and reproducibility of the assay procedure and time required for analysis. Table 1.2 summarizes chemical pollutants and corresponding human body fluids currently amenable to biological monitoring.

Strategies for detecting genotoxic and carcinogenic hazards associated with occupational chemical exposure should be incorporated within industrial monitoring programmes. These should include both in vitro and in vivo test systems (Brusick 1983). In vitro means of assessing cytogenetic effects involve both bacterial and mammalian cell culture systems (Green 1978). Available short-term genetic tests are presented in Table 1.3.

In vivo systems generally involve chronic exposure of animals,

TABLE 1.2

Pollutants and human sampling currently amenable to biological monitoring.

Pollutants	Biological Specimens		
	Blood	Urine	Expired Air
Arsenic	X	X	
Cadmium	X	X	
Chromium		X	
Lead	X	X	
Inorganic mercury	X	X	
Methyl mercury	X		
Carbon monoxide	X		X
Organochlorine pesticides	X		
Pentachlorophenol		X	
Polychlorinated biphenyls	X		
Chlorinated solvents	X	X	X
Benzene	X	X	X

from Berlin, Wolff, Hasegawa (1979)

TABLE 1.3

Short-term genetic test systems.

System	Tests Available
1. Bacterial	
1. <i>Salmonella typhimurium</i>	arabinose resistance (forward mutation) 8-azaguanine resistance (forward mutation) histidine reversion DNA repair assay
2. <i>Escherichia coli</i>	WP ₂ -tryptophan reversion 343/113-arginine, nicotinic acid reversion - galactose utilization (forward mutation) - gal ⁺ -lysine requirement (deletion)
3. <i>Bacillus subtilis</i>	rec ⁻ - DNA repair
2. Insect	
1. <i>Drosophila melanogaster</i>	sex-linked recessive lethals
3. Mammalian Cells in Culture	
1. Chinese hamster cells	HGPRT system-8-azaguanine resistance sister chromatid exchanges
2. Human leukocytes	sister chromatid exchanges unscheduled DNA synthesis
3. Human fibroblasts	unscheduled DNA synthesis

from Epler et al. (1980)

usually rodents, to chemicals, evaluation of risk based on incidence of cancers observed. Although providing analogous models for evaluation of work-related genotoxicity, obvious limitations in extrapolations to effects in man are inherent. Sensitivity to genetic lesions induced may differ between the animal species and man, time and cost associated with assessing the reproducibility of studies generally prohibitive. Combination of traditional *in vivo* studies with short-term genetic tests, as described by Green (1978), may provide a more reliable means of investigating cytogenetic effects of chemical exposure.

However, *in vivo-in vitro* systems involving analysis of cell culture systems and body fluids derived from exposed individuals may be a more viable strategy (Brusick 1983). Examples of tests, including sister chromatid exchange (SCE) analysis (Anderson et al. 1981; Conner et al. 1980; Garry et al. 1982; Kinsella & Radman 1978), and assessment of unscheduled DNA repair (Pero et al. 1981; Pero et al. 1982) currently applicable to human monitoring are given in Table 1.4. Use of these test systems may identify threshold doses or exposures capable of inducing genotoxic lesions, thereby enabling accurate assessment of proposed 'safe' or acceptable limits of chemical exposure within the workplace.

Industrial monitoring programmes should incorporate epidemiological assessments, involving reproductive, mortality and morbidity studies, evaluation of worker exposure, using both biological and environmental monitoring strategies, and cytogenetic monitoring of workers (Kilian & Picciano 1979; Legator & Rinkus 1979). Valid assessments of work-related health risks should only be made after integration of results achieved with each approach.

TABLE 1.4

Examples of in vivo/in vitro techniques used to identify genotoxic chemicals within industry.

Method	Target Cell Employed	Genetic Endpoint Assessed	Application to Human Monitoring
<p>1. Chromosomal analysis Expose animals in vivo, collect and culture in vivo blood. Cells arrested in metaphase are evaluated for structural and numerical chromosome damage.</p>	Circulating lymphocytes	Clastogenicity	Currently applied to human monitoring
<p>2. Sister chromatid exchange Same as above, except that Brd U is administered to the in vitro cultures prior to metaphase arrest. Sister chromatid exchange (SCE) is evaluated in M2 cells.</p>	Circulating lymphocytes	Direct chemical/DNA interaction	Currently applied to human monitoring
<p>3. Mutagenicity testing Expose animals in vivo, collect urine. Urine can be tested directly as a mutagen in an in vitro assay. Urine may also be concentrated and/or deconjugated prior to use in vitro.</p>	In vitro assays ranging from bacteria to cultured rodent or human cells	Only indicates that genotoxic agent has been formed in vivo and excreted via bladder	Currently applied to human monitoring

from Brusick (1983)

3. Role of Metabolism in Chemical Toxicology

Environmental pollutants absorbed by either inhalation of vapours or dermally (Maibach & Anjo 1981; Lauwerys et al. 1978) are eliminated in urine, expired air and faeces. The majority of occupational chemicals, being liposoluble, would be excreted very slowly due to accumulation in adipose tissue or reabsorption from renal tubuli (van Doorn et al. 1981b), if eliminated in unchanged form. Consequently, most chemicals undergo biotransformation, principally within the liver, generating polar metabolites rapidly cleared by the kidneys and eliminated in urine.

The first stage in metabolism of many foreign agents involves the microsomal mixed-function monooxygenases, an enzyme system located within the smooth endoplasmic reticulum. Oxidative metabolism by the microsomal monooxygenases may generate reactive electrophilic intermediates, such as epoxides (Pagano et al. 1982; Sims 1980), capable of covalently binding to nucleophilic sulphhydryl, amino and hydroxyl groups of cell protein, RNA and/or DNA. Alkylation of cell macromolecules by electrophilic metabolites is the primary step in initiation of cell lesions. Toxic effects of chemicals are therefore in many cases a consequence of metabolic activation rather than a direct action of the parent chemical (Toftgård et al. 1980).

Potent toxic/carcinogenic effects of some chemicals have been attributed to formation of 'bay region' epoxides (Jerina & Daly 1974). For example, carcinogenicity of benzo(α)pyrene, a ubiquitous environmental contaminant, is attributed to microsomal monooxygenase-catalyzed formation of a 9,10-epoxide metabolite, a 'bay region' diol epoxide, from the 7,8-dihydrodiol of benzo(α)pyrene (Guenthner & Oesch 1981; Tsang & Griffin 1979). Formation and irreversible binding of

an epoxide of vinyl chloride to cell nucleophiles has been shown to be responsible for potent carcinogenic properties of vinyl chloride monomer (Wisniewska-Knypl et al. 1981).

Increases in the activity of the microsomal monooxygenases, stimulated by exposure to anaesthetic gases, drugs such as anti-inflammatory agents, barbiturates and CNS stimulants, steroids, pesticides and environmental pollutants (Conney 1967) may be of major importance in influencing the metabolic fate of industrial chemicals. Cigarette smoke, containing a range of known carcinogens and mutagens including benzo(α)pyrene (van Doorn et al. 1981b), has been shown to enhance selective microsomal monooxygenase activities in both man and rats (Cooksley et al. 1979; Van Cantfort & Gielen 1981).

4. Role of Glutathione-S-Transferase and Other Detoxifying Enzymes

However, production of an electrophilic intermediate may not necessarily result in initiation of cell toxicity. Detoxification mechanisms within the cell, principally phase II reactions (Mulder 1979), are capable of inactivating these intermediates. Glucuronidation, sulphation, glutathione conjugation, acetylation, methylation and glycine conjugation are the most important reactions in man (Mulder 1979). Although these enzymes are mainly involved in detoxification, suggested roles in metabolic activation of some carcinogens such as aromatic amines have been proposed (Sims 1980).

The two major cellular protective mechanisms involve conjugation with glutathione, catalyzed by glutathione-S-transferase, and hydration to glycols or dihydrodiols by microsomal epoxide hydrolase (Pacifichi & Rane 1983).

Conjugation with reduced glutathione (GSH), a sulphhydryl-containing

tripeptide, catalyzed by glutathione-S-transferases (GSht) a group of enzymes with broad, overlapping specificities (Habig et al. 1974), is an efficient means of preventing metabolite-mediated toxicity (Jakoby 1978). Reduction in hepatic GSH concentration by administration of various hepatotoxins has been shown to precede development of hepatic lesions (Mitchell & Jollow 1975; Mitchell et al. 1976), suggesting GSH-dependent thresholds for metabolite-mediated toxicity.

Glutathione conjugates are further metabolized by cleavage of glutamate and glycine from the peptide side chain, catalyzed by gamma-glutamyl transferase and cysteinylglycinase, respectively (Meister 1975). Acetylation of the free amino group of the cysteine moiety generates N-acetylcysteine derivatives (mercapturic acids or thioethers) readily excreted in urine (Chasseaud 1979). Glutathione conjugation is the dominant detoxifying pathway in most tissues for many chemicals (Van Anda et al. 1979; Yashikawa 1980).

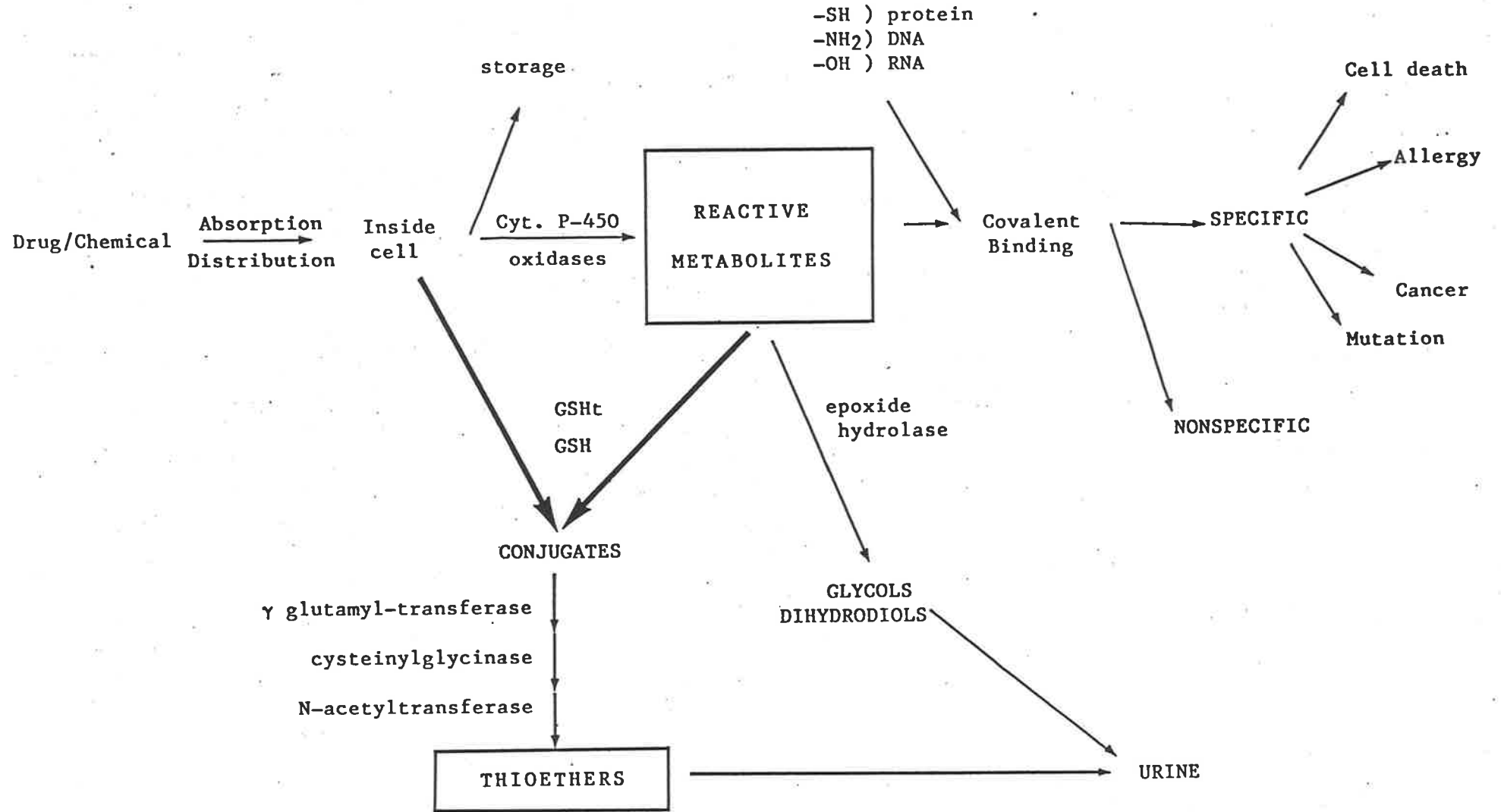
An alternative detoxification mechanism involves hydration of the epoxide by epoxide hydrolase. However, as previously discussed for polycyclic hydrocarbons, representative benzo(α)pyrene, hydration of epoxides may sometimes be the initial step in formation of ultimate carcinogens. An overview of major mechanisms involved in metabolism of environmental chemicals is shown in Diagram 1.1.

Availability of hepatic GSH and activity of GSht are important factors influencing the efficiency of cellular protective mechanisms against damage by alkylating intermediates.

Glutathione turnover rates may be modified by a variety of factors including nutritional status, disease states, stress, low dietary protein, drugs such as phenobarbital, ethanol and various hepatocarcinogens (Mainigi & Campbell 1981). Normal GSH status is maintained

DIAGRAM 1.1

A summary of major pathways of metabolism and elimination of environmental chemicals.



by feedback inhibition, reductions in the hepatic GSH pool stimulating de novo synthesis (Lauterburg et al. 1982). However, stressful treatments such as fasting and/or exposure to toxic chemicals may disrupt control (Brooks & Pong 1981; Lauterburg et al. 1982). Suppression of GSH synthesis, irrespective of significant reductions in availability incurred by such treatments, may considerably impair capacities of detoxifying pathways.

Activity of GSht is increased by a variety of chemicals including phenobarbital, 3-methylcholanthrene, tetrachlorodibenzo-p-dioxin, 3,4 benzo(α)pyrene, polyhalogenated biphenyls and insecticides (Kraus et al. 1981). Felton et al. (1980) demonstrated induction of GSht activity following administration of polycyclic aromatic hydrocarbons. Exposure to the range of polycyclic hydrocarbons within the occupational environment may therefore offer increased protection against metabolite-mediated toxicity.

Environmental chemicals are therefore common substrates for both toxification and detoxification reactions, net effect dependent on the relative activity of each pathway (Jollow & Smith 1977).

5. Urinary Thioether Excretion

(1) Development of the thioether assay

Glutathione conjugation results in the formation of thioethers, excreted as cysteine conjugates, mercapturic acids and other thioethers in bile and urine (van Doorn et al. 1981b). Many chemicals ubiquitous within the workplace may be possible substrates of glutathione-S-transferase, consequently eliminated in urine as mercapturic acids or thioethers. Table 1.5 lists some chemicals widely used within industry and partially excreted as mercapturic acids.

TABLE 1.5

Examples of industrial chemicals partially excreted as mercapturic acids.

Chemical	Application
Acrylamide	Acrylic fibres
Acrylonitrile	Plastic, coatings
Allyl alcohol	Resins
Aniline	Manufacture of dyes, solvents
Benzene	Solvent
Benzyl chloride	Manufacture of dyes
Biphenyl	Heat transfer agent
Epichlorhydrin	Varnishes, lacquers
Ethylene dichloride	Solvent
Ethyl acetate	Textile, coatings
Glycidol	Solvent
Mesityl oxide	Solvent
Methyl acrylate	Plastic films
Phenyl glycidyl ether	Stabilizator
Styrene	Plastic, rubber, resins
Vinyl chloride	Plastic
Vinylidene chloride	Plastic
O-xylene	Solvent

from van Doorn et al. (1981b)

It has therefore been proposed by Seütter-Berlage & Henderson (1977) and van Doorn et al. (1979) that urinary thioether excretion is indicative of metabolic clearance via glutathione conjugation, and that quantitation thereof may offer a convenient, non-invasive means of monitoring occupational chemical exposure. Assessments of individual sources of chemical exposure are unnecessary, as the technique is representative of biological monitoring approaches.

The assay procedure is non-selective, determination of urinary thioether concentrations reliant on the Ellman reaction (Ellman 1959) in which 5,5'-dithiobis-(2-nitrobenzoic) acid is reduced by thiol groups, yielding one mole of 2-nitro-5-mercaptobenzoic acid per mole of thiol group. As the nitromercaptobenzoic anion generated has an intense yellow colour, thiol concentrations may be quantitated spectrophotometrically. Optimal reaction pH of between 8.0 and 9.0 is critical, variations in intensity of colour development above or below this range attributable to pH only, and independent of thiol concentration (Sedlak & Lindsay 1968).

Methods employed by Seütter-Berlage & Henderson (1977), Pentz (1978), Vainio et al. (1978), Kilpikari (1981) and Kilpikari & Savolainen (1982) did not attempt to distinguish different forms of thio compounds present in urine: mercaptans (R-SH, thiols), disulphides (R-S-S-R') or thioethers (R-S-R') (van Doorn et al. 1981b). Addition of ascorbic acid to stabilize thio compounds and acid denaturation of urinary proteins were performed prior to alkaline hydrolysis of samples and determination of urinary thioether levels. In all studies high background values and large interindividual variations of non-exposed subjects were demonstrated, despite corrections made for pre-existing concentrations of thio compounds. Values reported were in

the range of 20-40 $\mu\text{mol SH}/\text{mmol creatinine}$ for non-exposed individuals and 25-120 $\mu\text{mol SH}/\text{mmol creatinine}$ for subjects exposed to varying ambient concentrations of occupational chemicals (Vainio et al. 1978).

Procedures adopted by van Doorn et al. (1979) attempted to eliminate interference due to the presence of pre-existing high concentrations of cystine - present usually within a range of 21-131 $\mu\text{mol}/\text{mmol creatinine}$ - and other disulphides. Modifications incorporated included:

- extraction of acidified samples with ethylacetate, to separate cystine from thiols and thioethers present;
- evaporation of extract;
- reconstitution and reduction with sodium tetraborate, to eliminate other disulphides.

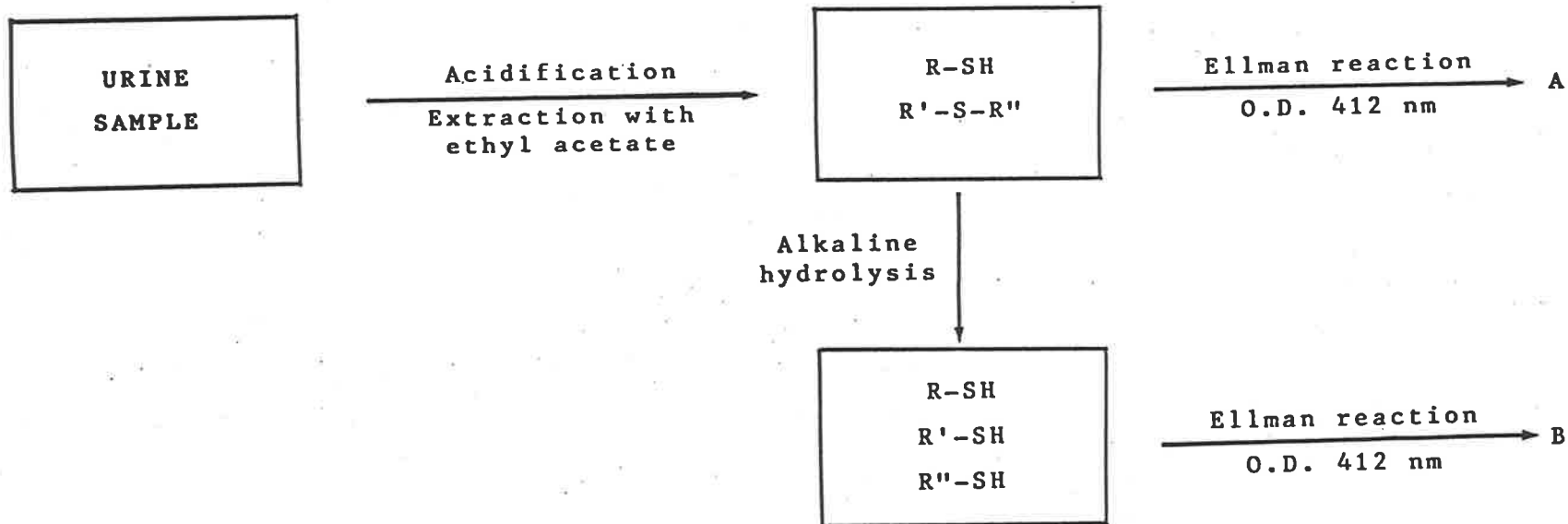
This reduction step was later omitted, as further corrections to account for the presence of other disulphides were deemed unnecessary (van Doorn et al. 1980). An outline of the assay protocol is provided in Diagram 1.2. Significantly lower background values were reported, interindividual differences of non-exposed subjects attributable to variations in cigarette consumption (van Doorn et al. 1979). Values reported ranged from 0.50 to 3.50 $\mu\text{mol SH}/\text{mmol creatinine}$ for non-exposed populations - dose-related increases reported for cigarette smokers - and 0.50 to 10.5 $\mu\text{mol SH}/\text{mmol creatinine}$ for operators of chemical waste incinerators (van Doorn et al. 1981a).

Urinary thioether concentrations were expressed relative to creatinine clearance to minimize variation due to fluctuations in urinary output.

Further developments in analysis and monitoring of urinary thioether excretion involve more selective approaches.

DIAGRAM 1.2

An outline of the assay protocol used for urinary thioether determination
(from van Doorn et al. 1981b).



Urinary Thioether Concentration = B-A

A method for quantitative determination of biological thiols, at picomole sensitivity, based on derivatization with monobromobimanes and separation by cation-exchange chromatography has been developed (Fahey et al. 1981). To date, the technique has only been applied to blood specimens, concentrations of various thiols including glutathione measured. However, further developmental work is required to eliminate fluorescent interference due to non-thiol impurities.

Buffoni et al. (1982) modified the assay procedure of van Doorn et al. (1979), incorporating both cation-exchange and reduction and affinity chromatographic separations, thereby eliminating interference due to cysteine and yellow urinary pigments, and free sulphhydryl and disulphide groups, respectively. Significant reductions in urinary thioether levels of non-exposed individuals were reported, range of 0.23-0.42 $\mu\text{mol SH/mmol creatinine}$.

Finally, high pressure liquid chromatographic analysis (HPLC) of isomeric thioether metabolites of styrene oxide has been demonstrated by Hernandez et al. (1982). Separations of ten different glutathione conjugates of styrene oxide were reported. Extension of the capabilities of this selective assay procedure, to incorporate the wide range of industrial chemicals, would greatly improve the utility of urinary thioether measurements, enabling sources of chemical exposure and associated health risks to be identified.

(2) Application of the thioether technique

(a) Previous studies

Previous studies by Vainio et al. (1978) and Kilpikari (1981) measured urinary thioether excretion of employees of chemical and rubber plants, based on associations of bladder and lung cancer with

long-term chemical exposure within both industries (Decoufle 1979). Enhanced thioether elimination by both radial tyre builders (Vainio et al. 1978) and workers handling uncured rubber (Kilpikari 1981) after a working day was reported. Both these groups were exposed to significant levels of chemicals, principally hexamethylene tetramine and resorcinol, by absorption via palmar skin or inhalation of airborne pollutants. Levels excreted were highly significant when compared with urinary thioether output of clerks of both factories.

Two studies involving chemical plant employees of waste incinerator operations (van Doorn et al. 1981a) and viscose production plants (van Doorn et al. 1981c) provided further evidence of elevation in urinary thioether output in response to occupational chemical exposure. Monitoring of ambient concentrations of carbon disulphide, an important component of viscose manufacture, and more selective determination of urinary elimination of carbon disulphide metabolites were both related to increased thioether excretion following a normal working day.

The most recent study, performed by Jagun et al. (1982) involved an assessment of urinary thioether elimination of nurses exposed to cytotoxic drugs, principally cyclophosphamide, vincristine and cytosine. It had previously been proposed that considerable drug absorption occurred during routine handling (Falck et al. 1979). Significant elevations in urinary thioether output by nurses after a working day, when taken in conjunction with reported mutagenicity of nurses' urine (Bos et al. 1982), provided further confirmatory evidence of cytotoxic drug absorption, either dermally or by droplet inhalation, a source of considerable health risk.

Longitudinal investigations of thioether output of rubber

plant employees showed initial decreases in work-related thioether excretion after one month's employment. However, enhanced thioether elimination in response to occupational chemical exposure was observed after five months' employment (Kilpikari and Savolainen 1982). Consequently, it has been suggested that monitoring of urinary thioether excretion within industry should only be carried out on workers after five to six months' continuous employment.

(b) Intended studies

Petroleum products, complex mixtures of lipophilic chemicals, are probably substrates of microsomal monooxygenases prior to elimination as polar metabolites, and may contain direct substrates of glutathione-S-transferases (Chasseaud 1979).

A previous study by Harman et al. (1981) demonstrated enhanced clearance of antipyrine, a metabolic probe drug, indicative of induced microsomal drug metabolism amongst petrol station attendants. However, attempts to relate induced metabolism of antipyrine with biological parameters of exposure proved negative.

Individuals involved in organic chemical research are exposed to a range of chemicals potentially noxious to health. Determination of proportions of chemicals absorbed, rather than ambient concentrations, may provide a more useful estimate of related health effects of chemical exposure (Gompertz 1980).

Fibreglass workers represent another occupational group in which organic solvent exposure is considerable. Styrene, the major pollutant of the workplace, undergoes biotransformation by the microsomal monooxygenases yielding styrene 7,8-oxide (Pagano et al. 1982). Evidence has been accumulated of mutagenic and genotoxic effects associated with this metabolite (Meretoja et al. 1977; Linnainmaa et

al. 1978; Busk 1979; Pero et al. 1982). Further metabolism by hydration, catalyzed by epoxide hydrolase, or conjugation, with glutathione result in urinary elimination of various metabolites including mandelic and phenylglyoxylic acids shown to be accurate indices of styrene exposure in both controlled laboratory and industrial situations (Bardoděj & Bardodějova 1970; Ohtsuji & Ikeda 1978; Engström et al. 1978b; Wolff et al. 1978) and mercapturic acid derivatives (Seutter-Berlage et al. 1978).

Consequently, application of the thioether technique within each occupational group may provide a rapid and viable means of monitoring intensities and biological effects of work-related chemical exposure.

6. Aims of this Thesis

The primary aim of this thesis was to evaluate the utility of the thioether technique as a biological monitor of chemical exposure. Validation of the assay and investigation of parameters modifying urinary thioether excretion were initially established using a suitable animal model. Administration of various chemicals differing in their extent of clearance via glutathione conjugation was designed to establish both sensitivity and reproducibility of the assay system.

Although nonspecific, the technique possesses several important advantages over current monitoring strategies, namely:

- non-invasive;
- simple;
- rapid.

The convenience of non-invasive urinary sampling from man was exploited in determining the effects of occupational chemical exposure on urinary thioether output.

C H A P T E R T W O
FACTORS MODIFYING URINARY THIOETHER
EXCRETION IN AN ANIMAL MODEL

CHAPTER 2

Introduction

A biological monitor of occupational chemical exposure must fulfil important characteristics of sensitivity, reliability and reproducibility of analysis before valid assessments of worker exposure can be made. Additionally, the technique must be both time- and cost-efficient, enabling routine applications within industry to be made.

Various workers have proposed that quantitation of urinary thioether excretion, indicative of clearance of xenobiotics or electrophilic metabolites via GSH conjugation, may offer a convenient non-invasive monitor of occupational chemical exposure (Seutter-Berlage & Henderson 1977; van Doorn et al. 1979). The major advantage of thioether determination is that it reflects absorption of chemicals capable of forming reactive metabolites irrespective of the mode of entry.

However, prior to application of the technique within industry, assessments of the validity of the assay system and investigation of parameters modifying urinary thioether output should be made using a suitable animal model. A previous study by Summer et al. (1979) attempted to validate the technique by administration of either diethylmaleate or naphthalene to rats and monkeys. Although dose-related increases in urinary thioether output were observed, no attempts to determine the sensitivity of the technique by titrating threshold doses required for production of detectable increases in thioether excretion were made.

This study aimed to evaluate both sensitivity and reproducibility

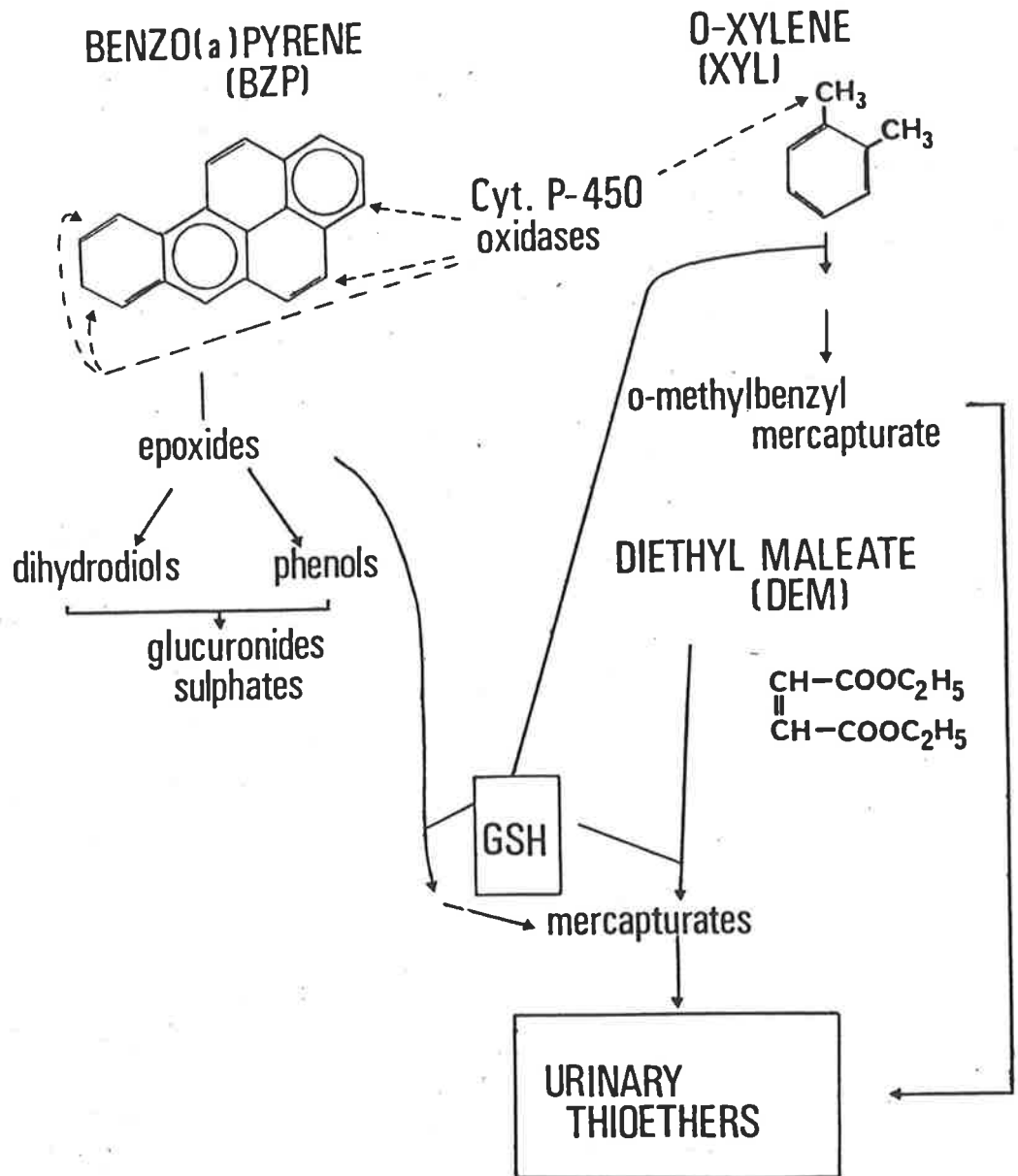
of the assay system by measuring urinary thioether concentrations eliminated by rats following dosage with three compounds substantially excreted in urine as mercapturates: benzo(α)pyrene (BZP), diethylmaleate (DEM) and o-xylene (XYL). Choice of this species was based on convenience and sampling practicalities, as large numbers were readily obtained and maintained and dosage and sample collection easily managed.

Substrates chosen differed considerably in both mechanism and extent of clearance via GSH conjugation. BZP, a representative of the class of ubiquitous environmental chemicals known as polycyclic hydrocarbons, is a potent mutagen and carcinogen (Morgenstern et al. 1981). Carcinogenicity is a result of production of reactive oxidative metabolites (including epoxides and dihydrodiol epoxides; Sims 1980) and consequent arylation of nucleophilic groups of cell macromolecules. However, reactive arylating intermediates may undergo detoxification by conjugation with GSH, subsequently eliminated in urine as mercapturic acid derivatives. DEM is a direct substrate for GSHT, and hence elimination is primarily due to this metabolism (Chasseaud 1979). Administration of XYL has been shown by van Doorn et al. (1980) to result in urinary elimination of o-methylbenzylmercapturic acid, implying epoxidation of the aromatic nucleus and consequent detoxification by conjugation with GSH. A summary of the relevant metabolic pathways of each substrate is presented in Diagram 2.1.

Urinary thioethers may therefore result from either direct conjugation of the chemical with GSH (e.g., DEM), or indirectly, by conjugation with a reactive metabolite generated by the microsomal monooxygenases. Selective induction of the microsomal system may enable distinctions to be made of the relative contribution of each

DIAGRAM 2.1

An overview of relevant metabolic pathways of the thioether substrates benzo(α)pyrene (BZP), o-xylene (XYL) and diethylmaleate (DEM).



mechanism to urinary thioether output.

The terminal component of the microsomal monooxygenase system, cytochrome P-450, exists in multiple forms differentially induced by chemicals. Stimulation of microsomal monooxygenase activity may be classified as either 'phenobarbital-like', as produced by phenobarbital (PB), or polychlorinated biphenyl (PCB), or '3-methylcholanthrene-like', representative agents being 3-methylcholanthrene (3-MC), BZP and beta-naphthoflavone (BNF) (Conney 1967; Madhukar & Matsumura 1981). Administration of PB has been shown to produce proliferation of the smooth endoplasmic reticulum and induction of at least three forms of cytochrome P-450 (Ryan et al. 1979), thereby increasing metabolism of a wide range of substrates. However, pretreatment with 3-MC results in a selective induction of two different forms of cytochrome P₁-450 (or P-448), thereby stimulating activity of only a narrow range of enzymes including aryl hydrocarbon hydroxylase (Negishi & Nebert 1981). Activities of GSH transferases are stimulated to different extents by both types of pretreatment regimes (Mukhtar & Bresnick 1976; Mukhtar et al. 1981).

Various factors including diet, alcohol and cigarette consumption, occupation and exposure to pesticides may influence the metabolic capacities of the individual (Cooksley et al. 1979). Cigarette smoke, containing numerous carcinogens and mutagens including BZP (van Doorn et al. 1981b), has been shown to enhance activity of aryl hydrocarbon hydroxylase and, concomitantly with occupational chemical exposure, may significantly alter xenobiotic metabolism. Treatment with microsomal inducers selective for cytochrome P-448-related enzymes prior to chemical dosage of rats may provide an analogous model for assessing possible interactive effects on thioether excretion by

occupational chemical exposure and cigarette consumption in man.

Methods

1. Animals

Adult male Porton rats, weighing 180-220 g, were obtained from the Waite Agricultural Research Institute, South Australia, and housed in the Adelaide University Central Animal House. Untreated animals were kept in a 12-hour light/dark cycle and allowed food (standard laboratory chow) and water ad libitum. Chemicals were administered in peanut oil as a single dose i.p. Time of administration was usually 0830 hours. Following drug administration, rats were housed in individual stainless steel metabolism cages with free access to food and water. Urine was collected daily and frozen until required for assay.

2. Pretreatment Regimes

The following inductive pretreatments were given:

(a) Phenobarbital (PB) administered in drinking water at a concentration of 1 mg/ml for a period of 7-10 days.

(b) Beta-naphthoflavone (BNF) single i.p. injection of 200 mg/kg 40 hours prior to drug administration.

(c) Polychlorinated biphenyl (PCB) single i.p. injection of 500 mg/kg 4-5 days prior to drug administration.

(d) Benzo(α)pyrene (BZP) single i.p. injection of 80 mg/kg 65 hours prior to drug administration.

(e) 3-methylcholanthrene (3-MC) single i.p. injection of 80 mg/kg 40 hours prior to drug administration.

3. Thioether Substrates

The following thioether substrates were administered as a single i.p. dose:

Benzo(α)pyrene (BZP)	40-320 $\mu\text{mol/kg}$
Diethylmaleate (DEM)	60-2320 $\mu\text{mol/kg}$
O-xylene (XYL)	50-500 $\mu\text{mol/kg}$

Urine was collected over two consecutive 24 hour periods after treatment.

4. Assays

(1) Urinary thioether

Urinary thioether concentration was determined as described by van Doorn et al. (1980), with slight modifications.

Thawed urine samples were centrifuged at 3000xg for five minutes before analysis. Aliquot samples of 1.0 ml of clear urine were transferred to stoppered extraction tubes and pH adjusted with 4N HCl to 1.5 to 2.0. After addition of 2.0 ml of ethylacetate, tubes were shaken vigorously for 15 minutes using a shaking apparatus. The layers were separated by centrifugation at 1000xg for five minutes. After removal of the ethylacetate layer, the extraction procedure was repeated with another 2.0 ml of ethylacetate. The collected ethylacetate layers were evaporated to dryness under nitrogen. The residue was reconstituted in 2.0 ml of distilled water.

Alkaline hydrolysis was performed on 1.0 ml samples in screw-capped tubes by the addition of 0.5 ml of 4N NaOH, saturation with nitrogen, and keeping the closed tubes at 100°C for 60 minutes. The tubes were then cooled on ice for 10 minutes. During mixing 0.5 ml of 4N HCl was added, and sulphhydryl (-SH) concentration determined

according to Ellman (1959).

A 5.0 ml aliquot of the aqueous solution was added to 4.0 ml of 0.1 M phosphate buffer, pH 7.5, containing 0.1 M EDTA and 0.60 ml of a 5,5'-dithiobis-(2-nitrobenzoic) acid solution (0.4 mg DTNB per ml of 1% sodium citrate solution) then added. Sulphydryl concentration was also measured prior to hydrolysis of the urine extract using this same assay procedure.

Absorbance was read at 412 nm on a Pye Unicam SP 1800 spectrophotometer. Corrections were made for the contribution of the extract and DTNB solution to the absorbance. Sulphydryl concentration was calculated from a standard curve using N-acetylcysteine, and accounting for efficiencies of extraction and hydrolysis procedures. (Refer to Appendix 1 for explanation of correction factors and standard curve involved in determination of sulphydryl concentration.) Thioether concentration of the sample was equal to the difference of the two sulphydryl concentrations (post-pre hydrolysis concentrations). Concentrations were expressed as μ mole thioether relative to creatinine concentration (mmoles) to minimize variations due to fluctuations in urine output.

(2) Creatinine

Creatinine concentration was determined by the method of Yatzidis (1974). (Refer to Appendix 2 for an outline of methodology involved.)

(3) Assay for hepatic glutathione (GSH) concentration

Rats were sacrificed after each inducing treatment at a time coincident with thioether substrate administration. This sampling time, corresponding to maximal inductive effect of each pretreatment,

was chosen in order to assess possible changes in GSH status produced by each treatment regime. Livers were perfused with ice-cold 0.9% saline and placed in ice-cold buffer (0.1 M, pH 7.4). Determination of GSH concentration was based on the colourimetric method of Ellman (1959). An extinction coefficient of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (Ellman 1959) was used in determination of hepatic GSH concentration.

(4) Assay for GSH-S-transferase activity (GSht)

(a) S-9 fraction preparation

Animals were sacrificed after each pretreatment regime at a time coincident with thioether substrate administration, between 0800 and 0900 hours, to minimize any diurnal variation in enzyme activity (Tredger & Chhabra 1977). This sampling time, corresponding to maximal inductive effects of each pretreatment, was chosen in order to assess possible changes in GSht activity resulting from each treatment regime. All subsequent operations were performed at $0-4^{\circ}\text{C}$.

Livers were excised, perfused with ice-cold 0.9% saline and placed in centrifuge tubes containing two volumes of phosphate buffer (0.1 M, pH 7.4). After weighing, livers were homogenized using an Ultra Turrax homogenizer (Janke & Kunkel KG, Staufen, WG) and centrifuged at 9000 r.p.m. ($10,400\text{xg}$) in a Beckman J2-21 centrifuge for 15 minutes. The resulting supernatant (S-9 fraction) was decanted and adjusted with buffer to a known concentration of liver.

(b) Assay for GSht activity

Conditions for the assay of S-9 fraction for GSH-transferase activity toward 1-chloro-2,4-dinitrobenzene (CDNB) were identical with those of Habig et al. (1974), except that the final concentration of

CDNB in the incubation mix was reduced to 100 μM . Assays were performed with a Pye Unicam SP 1800 spectrophotometer at 340 nm. (Refer to Appendix 3 for an outline of methodology involved.)

5. Data Analysis

The Mann-Whitney U-test for unrelated samples and Wilcoxon matched-pairs signed ranks test for related samples were used for analyzing differences in thioether excretion due to either dose or sampling period, respectively. Both Kruskal-Wallis one-way analysis of variance (Siegel 1956) and Mann-Whitney U-tests were employed in evaluating the statistical significance of the effects of inductive treatments on:

- (a) basal thioether output;
- (b) substrate-related thioether excretion;
- (c) hepatic GSH;
- (d) GSH transferase activity.

Results

1. Effect of Thioether Substrates

Urinary thioether output of untreated rats measured over a period of 7 days was consistently low, 0.44 $\mu\text{mol-SH/mmol}$ creatinine, s.e.m. of 0.01. All three thioether substrates produced significant dose-related increases in this output. (Refer to Table 2.1, Mann-Whitney U-test, two-tailed, $P < 0.001$). Concentrations excreted after highest substrate dosage were 40 (BZP), 284 (DEM) and 183 (XYL) $\mu\text{mol-SH/mmol}$ creatinine, representing increases of 90, 644 and 415-fold, respectively.

TABLE 2.1

Urinary thioether elimination by the rat 24 and 48 hours following administration of benzo(α)pyrene (BZP), diethylmaleate (DEM) and o-xylene (XYL).

Treatment	Dose ($\mu\text{mol}/\text{kg}$)	Urinary Thioether ¹ ($\mu\text{mol-SH}/\text{mmol creatinine}$)	
		24 hours	48 hours
Control	-	0.44 (0.01)	0.44 (0.01)
BZP ^c	40	1.17 (0.02)	
	80	4.52 (1.56)	1.82 (0.15) ^a
	160	12.5 (2.73)	4.84 (0.68) ^a
	320	40.0 (5.00)	16.2 (0.96) ^a
DEM ^c	60	30.1 (4.34)	
	120	48.7 (10.1)	
	290	112 (7.45)	20.6 (2.54) ^b
	580	136 (10.7)	27.6 (1.64) ^b
	1160	190 (24.5)	47.1 (1.13) ^b
	2320	284 (22.5)	
XYL ^c	50	7.16 (0.67)	
	100	22.9 (2.38)	
	250	104 (3.56)	15.1 (1.47) ^b
	500	183 (7.61)	29.2 (2.78) ^b

1 Results are given as mean (\pm s.e.m.), n = 6, except control where n = 20.

a,b Significantly different from 24 hour sample, Wilcoxon matched pairs signed rank test, two-tail, $P < 0.005$, $P < 0.001$.

c Significantly different from control, Mann-Whitney U, two-tail, $P < 0.001$.

Thioether output was significantly lower in the second 24-hour sample after dosage with each substrate (Wilcoxon matched-pairs signed rank test, $P < 0.005$, $P < 0.001$). In the case of DEM and XYL, values obtained in the second 24-hour sample were 5 to 6-fold lower than levels excreted in the first 24-hour sample, while thioether concentrations measured in the second 24-hour sample of rats given BZP were only 2 to 3-fold lower.

2. Effect of Pretreatments

Induction of microsomal enzyme activity by pretreatment with both cytochrome P-448 specific and general enzyme inducers raised thioether output in both control and rats treated with BZP, DEM and XYL (refer to Diagram 2.2). Greatest increases of a range of 4 (PB)- to 30 (BZP)-fold magnitude were measured in BZP-dosed rats. Pretreatment with BZP produced the largest increase in both baseline and chemical-related thioether excretion (Mann-Whitney U-test, $P < 0.001$).

All inductive treatments produced significant increases in GSH-transferase activity (Kruskal-Wallis one-way analysis of variance, Mann-Whitney U, $P < 0.005$, $P < 0.001$; refer to Table 2.2). Similar changes have been reported by Mukhtar & Bresnick (1976) and Felton et al. (1980). However, only two treatments (PB increased $P < 0.01$, BZP decreased $P < 0.005$) produced significant changes in hepatic GSH concentration from control levels. Changes in hepatic GSH concentration were minor in relation to the changes in GSH-transferase activity, and small when considered as a percentage of basal levels.

DIAGRAM 2.2

The effect of microsomal enzyme induction on basal (▨) and substrate-related (□) urinary thioether excretion of rats. Values shown are mean \pm s.e.m. (n = 6). Values in parentheses represent the magnitude of increased thioether excretion after accounting for inductive pretreatment effects on basal thioether excretion.

THIOETHER ($\mu\text{mol}\cdot\text{SH}/\text{mmol creatinine}$)

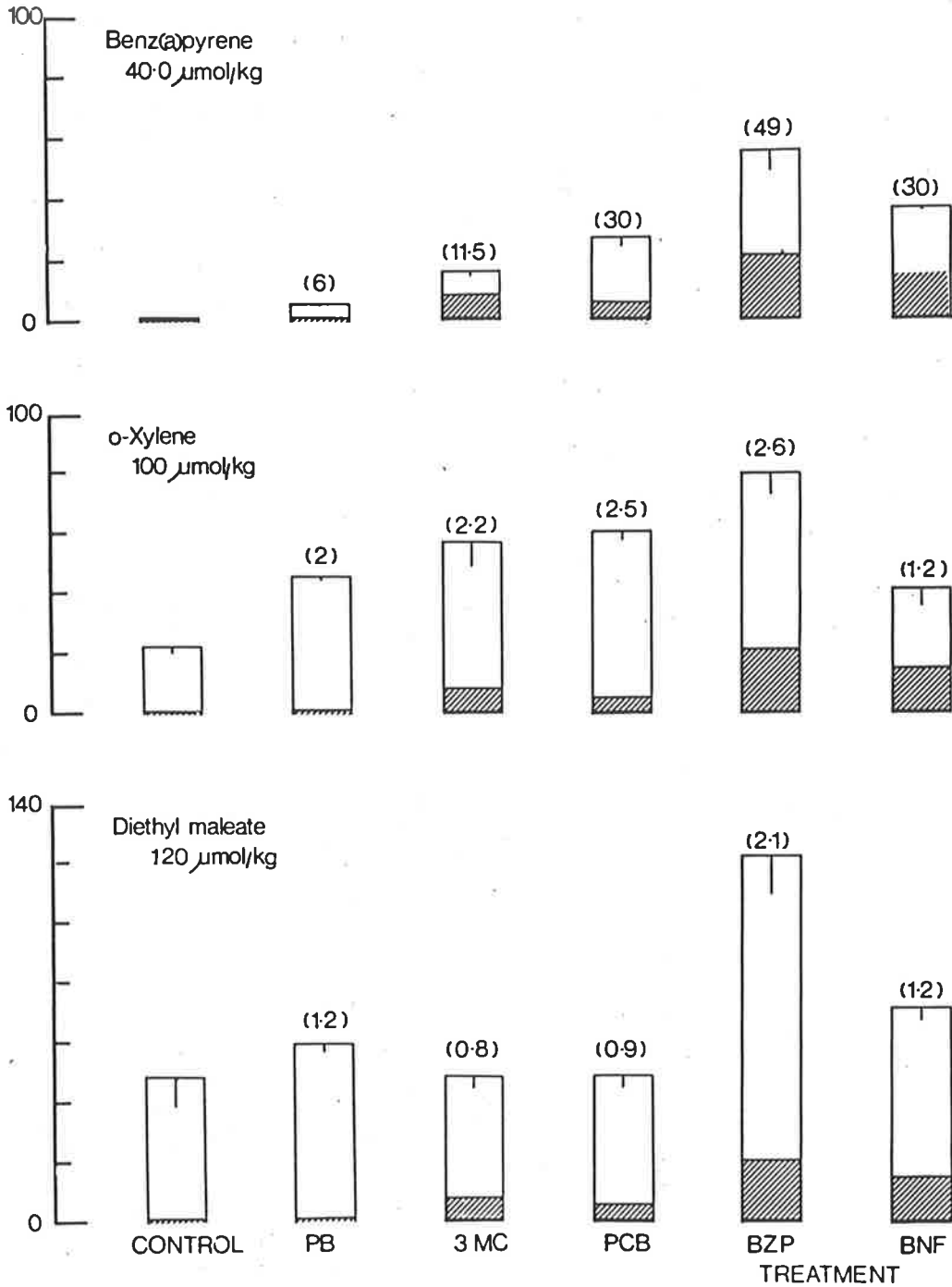


TABLE 2.2

Effect of microsomal monooxygenase induction on hepatic glutathione (GSH) concentration and GSH-transferase (GSht) activity.

Treatment	GSH ¹ ($\mu\text{mol/g liver}$)	GSht ¹ ($\mu\text{mol conjugate/}$ min/g liver)
Control	8.79 (0.19)	78.4 (4.22)
PB	9.68 (0.20) ^a	195 (17.9) ^c
PCB	9.25 (0.38)	110 (4.58) ^b
3-MC	8.58 (0.28)	155 (2.72) ^c
BZP	7.53 (0.23) ^b	145 (2.82) ^c
BNF	9.16 (0.37)	146 (3.28) ^c

Results are given as mean (\pm s.e.m.), n = 6.

Significantly different from control, Mann-Whitney U-test, two-tail.

a P<0.01

b P<0.005

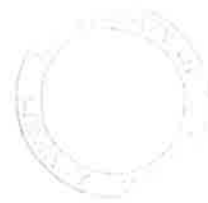
c P<0.001

Discussion

In this study urinary thioether quantitation fulfilled the criteria of sensitivity, reproducibility and non-invasive convenience, necessary requirements of a biological monitor of chemical dosage or exposure (Zielhuis 1979). Basal urinary thioether output of rats was consistently low and significantly enhanced in a dose-related fashion after administration of each chemical. On a comparative basis, the rat basal thioether output is lower ($0.44 \mu\text{mol-SH}/\text{mmol creatinine}$) than that excreted by non-exposed humans (range of 0.50 to $3.00 \mu\text{mol-SH}/\text{mmol creatinine}$), but the treatment induced output (1.0 to $300 \mu\text{mol-SH}/\text{mmol creatinine}$) encompasses the range of thioether concentrations excreted by man following exposure to chemicals in the occupational environment (Stock & Priestly 1982; van Doorn et al. 1979; van Doorn et al. 1981a).

Although dose ranges for all three substrates were comparable, thioether output following administration of DEM and XYL was significantly greater, indicating a greater proportion of each substrate cleared via GSH conjugation. Both DEM- and XYL-related thioether excretion was rapid, predominantly within the first 24 hours after dosage. However, reduction of thioether output to basal levels after a single dose of BZP was attenuated; concentrations measured within the second 24-hour sample were approximately half of maximum levels excreted. Therefore, while urinary thioether output may be a useful guide to recent exposure for some chemicals, it is not uniformly so, as pharmacokinetic and pharmacodynamic factors may influence both levels and rates of thioether output.

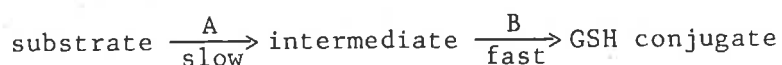
Although evidence has been presented in this study supporting the



proposal that urinary thioether quantitation may represent a useful monitor of chemical exposure, the technique does possess certain limitations, which will be fully discussed in Chapter 5, in relation to interpretation of data. Of primary consideration, however, is the nonspecificity of the technique and consequent inability to distinguish mercapturates arising from direct or GSH-transferase-mediated conjugation of substrates with GSH directly, or indirectly with electrophilic oxidative metabolites. Analysis of the effects of enzyme-inducing pretreatments, particularly those relatively selective for cytochrome P-450 isozymes such as cytochrome P-448, may possibly facilitate differentiation of the mechanism of urinary thioether output associated with administration of each substrate.

In this study, after accounting for elevation of basal thioether output associated with the enzyme-inducing pretreatments alone, it was shown that both BZP- and XYL-related thioether excretion were sensitive to both general (PB and PCB) and cytochrome P-448-selective (3-MC, BZP, BNF) inducers. BZP-associated thioether elimination was particularly sensitive to induction by cytochrome P-448-specific inducers, a maximum increase of 30-fold above control levels being observed. DEM-related thioether excretion was only sensitive to BZP induction.

Explanation of these effects can be made on the basis of reaction kinetics using the proposed reaction sequence:

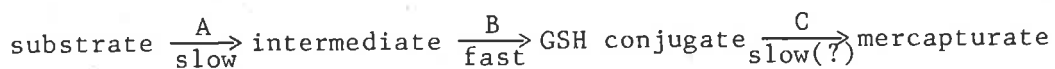


Using this model, thioether output associated with chemical dosage should be quite insensitive to changes in reaction B (changes in GSH-transferase activity), but, however, acutely sensitive to changes in formation of reactive oxidative metabolites (reaction A). Reaction A could possibly be viewed as the 'rate-limiting step' of the sequence

and consequently a major determinant of urinary thioether output.

Experimental data provides support for the proposed reaction sequence. Thioether output associated with both BZP and XYL - both metabolized to an extent by the microsomal system - was significantly increased following enzyme induction. As each pretreatment (particularly PB) induced GSH-transferase activity, enhanced thioether output could not be attributed entirely to changes in the formation of oxidative electrophilic metabolites. However, elevation in DEM-related thioether excretion was slight following enzyme induction, inferring that the influence of GSH-transferase activity may be small in comparison to changes in the provision of oxidative metabolite substrates for the enzyme.

Increased thioether output associated with DEM dosage tends to contradict this model, suggesting instead that the reaction sequence is sensitive to changes in GSH-transferase activity. Modification of the proposed reaction sequence may, however, explain this result. GSH-transferase, as suggested by experimental results, is probably not 'rate-limiting', but instead activity of a subsequent stage of metabolism, possibly involving conversion of a GSH conjugate(s) to mercapturates, may therefore influence urinary thioether excretion. Incorporating this modification, the reaction sequence involves the following steps:



Chemical-related thioether output is mainly determined, therefore, by the activity of microsomal monooxygenases and consequent availability of oxidative electrophilic metabolites, substrates for GSH-transferase. Both activities of GSH-transferase and enzymes involved in mercapturate formation have minor influence on urinary thioether excretion.

As DEM is thought to conjugate directly with GSH, it may have been expected that the pretreatment producing the greatest increase in GSH-transferase activity (PB) may have produced the greatest increase in DEM-associated thioether output. The three following conjectures, based on experimental findings, may jointly explain this anomalous result.

Firstly, the possibility that a BZP-inducible metabolite also contributes to DEM-associated thioether output cannot be discounted. Secondly, inducible changes in GSH-transferase activity towards the substrate CDNB may not reflect changes in conjugative activity towards other substrates. Finally, while the biliary/faecal output of DEM-derived thioethers may be smaller than the corresponding urinary output (Rozman et al. 1982), modifications of the biliary/urinary balance may provide explanations for changes observed.

Xenobiotics are usually metabolized within the liver by several pathways, induced to differing extents by each pretreatment (Jollow & Smith 1977). Consequently, the overall effect of enzyme induction is a result of summation of inductive effects on each pathway. Whilst in these experiments inducers, including PCB, enhanced thioether output from oxidizable substrates, Heinonen et al. (1982) reported a PCB-induced reduction in thioether output associated with vinyltoluene inhalation. This effect was attributed to a selective enhancement of the alternative detoxification pathway, mediated by epoxide hydrase. Therefore, the importance of relative induction of pathways other than those leading to mercapturates should not be underestimated when interpreting chemical-related thioether data.

C H A P T E R T H R E E
URINARY THIOETHER ELIMINATION BY CONTROL
AND OCCUPATIONALLY-EXPOSED POPULATIONS

CHAPTER 3

Introduction

Experimental studies in Chapter 2 showed that thioether output was increased in a dose-related manner following chemical administration or exposure. Thioether output was sensitive to both small doses of chemicals metabolized to an appreciable extent by GSH conjugation (e.g., DEM) and induction of microsomal monooxygenase activity, the proposed 'rate-limiting step' of the reaction sequence suggested in Chapter 2. Therefore, quantitation of urinary thioether excretion may offer a useful alternative method of monitoring occupational chemical exposure, as opposed to current approaches in which ambient chemical or pollutant concentrations are assessed. The technique may provide a more accurate assessment of dose or exposure load, accounting for interindividual differences in xenobiotic absorption and metabolism, and consequently may be a more realistic indicator of bioavailability of chemicals in the working environment.

The practicality of this monitoring approach has been investigated by other workers in a variety of occupational situations. Studies by Vainio et al. (1978) and Kilpikari (1981) measured urinary thioether elimination of workers of both chemical and rubber plants following a normal working day. Further applications of the technique involved operators of chemical waste incinerators (van Doorn et al. 1981a), employees of polyviscose production factories (van Doorn et al. 1981c), and nurses routinely involved in handling cytotoxic drugs (Jagun et al. 1982). Each working situation was thought to be a source of potential health risks to individuals concerned, based on epidemiological reports of increased incidence of cancer and

tumours amongst workers (particularly rubber and chemical plant workers), and offered the potential for significant exposure to chemicals capable of undergoing bioactivation by the microsomal mono-oxygenase system.

The susceptibility of the microsomal enzymes to both acquired and inherited variability is well known. Both environmental pollutants, such as pesticides, and components of the individual's lifestyle including dietary factors, cigarette and alcohol consumption and caffeine intake, have been shown to modify microsomal enzyme activity (Brodie et al. 1981). Additionally, various drug treatments such as anticonvulsants, barbiturates and steroids are known to stimulate microsomal monooxygenase metabolism. Response of the individual to these agents is, to an extent, genetically determined. Changes in microsomal xenobiotic metabolism, a major factor influencing the availability of substrates for GSH-transferase and hence thioether output, may become more critical in working situations involving exposure to certain organic solvents previously shown to reduce the capability of detoxifying pathways in man (Dolara et al. 1982).

The relationship between exposure period and time of sampling is a factor which has previously been given relatively little consideration. Earlier studies, although demonstrating work-related increases in urinary thioether excretion, did not account for differences in chemical elimination due to kinetic properties, relying instead on 'spot' sample collections either preceding or on completion of the work shift. Considerations of the pharmacokinetics of chemicals present in the working environment are, however, necessary in designing a suitable sampling strategy. Monitoring

exposure to chemicals with particularly short biological half-lives may require more frequent sampling, whereas exposure to chemicals with relatively slow elimination kinetics (such as lipophilic organic agents) may necessitate sampling over longer time intervals and require assessments of chemical or metabolite levels present prior to commencement of the next work shift.

The primary aim of this study was to evaluate the thioether technique by application within selected occupational situations thought to offer the potential for significant exposure to chemicals, possible substrates - either directly or via metabolite formation - of GSH-transferases. The effect of various sources of variation, including dose (or exposure level), sampling time and concurrent chemical exposure, such as smoking or alcohol intake, on urinary thioether output was investigated. Initially, urinary thioether elimination of a healthy 'non-exposed' population - individuals not exposed to appreciable chemical levels during workday routines - was monitored over a 24 hour period, in an attempt to determine if diurnal fluctuations in urinary thioether elimination were evident. The technique was then applied within two different occupational groups - petrol station attendants and organic chemistry research staff - in which the potential for chemical exposure was expected to be relatively heavy.

Petrol station attendants are thought to receive relatively heavy exposure to petroleum products, complex mixtures of lipophilic chemicals, in comparison to the general public. A previous study by Harman et al. (1981) demonstrated enhanced clearance of the metabolic probe drug, antipyrine, by petrol station attendants, attributed to microsomal enzyme induction produced by petroleum inhalation.

However, attempts to relate the rate of antipyrine clearance to biological parameters of exposure, such as urinary phenol excretion or plasma/urinary lead levels, proved unsuccessful due to the variability and relative insensitivity of these methods for detecting petroleum absorption.

Organic chemistry laboratory staff are exposed to a wide range of chemicals during the working day, with the potential for bio-activation by, and stimulation of, the microsomal monooxygenase system. Cytogenetic monitoring of chemical workers has revealed significant genotoxicity, detected by either increases in sister chromatid exchange (SCE) or modifications of the DNA repair systems. Induction of unscheduled DNA synthesis, an index of DNA damage, was demonstrated following long-term occupational exposure to organic solvents (Frost & Legator 1982).

The utility of the thioether technique in assessing intensity of chemical exposure in each occupational group was also evaluated.

Methods

1. Subjects

(1) Controls

Twenty-five healthy individuals, seventeen male and eight female, volunteered to act as controls in these experiments. None of these were taking any medication at the time of the study. The ages of males ranged from 18 to 37 years (25 ± 6 , mean \pm SD). The females ranged in age from 19 to 25 years (23 ± 2 , mean \pm SD). The group comprised teachers, office and laboratory staff whose daily work routine did not suggest any appreciable potential for chemical exposure.

(2) Research laboratory staff

Twelve male and one female employee of the Department of Organic Chemistry, University of Adelaide, South Australia, participated in the study. All subjects had worked in organic chemistry research laboratories for at least one year. The range of chemicals to which subjects were exposed included solvents (e.g., ethers, petroleum products), halogenated chemicals (e.g., carbon tetrachloride, dichloromethane), pyridines and heavy metals. The period of exposure varied from less than one hour to an eight hour working day exposure. Subjects were aged 19 to 55 years (28 ± 9 , mean \pm SD).

(3) Petrol station attendants

Forty-three male and five female employees at 25 suburban petrol-vending stations participated in the study. Thirteen subjects from this population were employed in self-serve outlets. All subjects had worked as petrol pump attendants or garage mechanics, or both, for more than one year. They were aged 16 to 53 years (29 ± 10 , mean \pm SD).

2. Sampling Protocol

(1) Controls

Volunteers were asked to collect total urine excreted during a working day in four six-hourly samples. Urine samples were frozen until assayed.

(2) Research laboratory staff

Subjects were asked to collect two urine samples on both a non-working day (Sunday) and mid-week working day. Morning samples were collected between 0700 and 0800 hours and evening samples collected between 1900 and 2000 hours. Samples were frozen after collection

and stored frozen until analysis.

(3) Petrol station attendants

Subjects were asked to collect two urine samples on a mid-week working day, one pre-work sample collected between 0600 and 0700 hours and one post-work sample collected between 1900 and 2000 hours. Samples were frozen after collection until analysis.

A short questionnaire giving details of diet, medications taken, cigarette consumption and employment duties was completed by each participant at the time of sample collection. A copy of the questionnaire used in this study is included in Appendix 4.

3. Assay of Thioether and Creatinine

Urinary thioether and creatinine concentrations were determined as previously described (refer to Chapter 2, Methods).

4. Data Analysis

Non-parametric statistical procedures were preferred to obviate the need for assuming the nature of the data distribution. The Wilcoxon matched-pairs signed-ranks test for related samples was used for analysis of differences in thioether excretion due to sampling time. Statistical significance of smoking-related increases in thioether output was evaluated using the Mann-Whitney U-Test for unrelated samples.

The one-sample runs test was used to determine whether distribution of thioether data was random with respect to cigarette or alcohol consumption, or workshop duties (for petrol station attendant data). Finally, the significance of possible interactive effects between either cigarette consumption or workshop duties and occupational

chemical exposure was investigated using both an analysis of variance and Chi square tests for k independent samples (Siegel 1956).

Results

Table 3.1 shows urinary thioether excretion over 24 hours of twenty-five volunteers that participated in the study. Thioether output of each individual was a mean value of four six-hourly samples. Values determined in this population (mean of 1.57 $\mu\text{mol-SH}/\text{mmol}$ creatinine, range of 0.49 to 3.75 $\mu\text{mol-SH}/\text{mmol}$ creatinine) were consistent with findings reported by van Doorn et al. (1979) in a similar study. Urinary thioether output was uniformly low and showed no significant diurnal variability (refer to Diagram 3.1). Thioether elimination was significantly greater in smokers than non-smokers (Mann-Whitney U-test, $P < 0.001$), a finding consistent with similar observations made by van Doorn et al. (1979).

Some research laboratory staff excreted higher levels of thioethers in evening urine samples compared to morning urine samples, and for the group as a whole, the changes were statistically significant on working days only. (Refer to Table 3.2, Wilcoxon matched-pairs signed-ranks test, $P < 0.01$, two-tail). Elevation in urinary thioether output varied with both the nature and intensity of chemical exposure, highest work-related increase of 19.6 $\mu\text{mol-SH}/\text{mmol}$ creatinine associated with an eight-hour exposure to halogenated organic solvents.

Evaluation of data obtained from petrol station attendants indicated that the type of petrol retailing operation markedly influenced urinary thioether output. Individuals employed in pump-

TABLE 3.1

Twenty-four hour urinary thioether elimination of
17 male and 8 female volunteers (control study).

Subject	Sex	Age (yrs)	S/NS ¹	Thioether ² ($\mu\text{mol-SH}/\text{mmol}$ creatinine)
1	M	21	NS	0.49 (0.01)
2	M	21	NS	0.63 (0.01)
3	M	21	NS	0.71 (0.01)
4	M	20	NS	0.71 (0.08)
5	F	22	NS	0.75 (0.03)
6	F	25	NS	0.77 (0.06)
7	F	25	NS	0.77 (0.06)
8	F	22	NS	0.80 (0.10)
9	M	20	NS	1.00 (0.07)
10	M	37	NS	1.08 (0.19)
11	M	27	NS	1.12 (0.10)
12	F	22	S	1.12 (0.32)
13	M	18	S	1.53 (0.33)
14	M	21	S	1.59 (0.33)
15	M	22	S	1.69 (0.19)
16	M	25	S	1.70 (0.09)
17	M	24	S	1.78 (0.33)
18	F	19	S	1.99 (0.20)
19	M	33	S	2.02 (0.04)
20	M	20	S	2.17 (0.44)
21	F	23	S	2.41 (0.20)
22	M	31	S	2.77 (0.58)
23	M	27	S	2.84 (0.66)
24	M	34	S	3.07 (0.78)
25	F	25	S	3.75 (0.59)

1 S = smoker, NS = non-smoker.

2 Thioether given as a mean value of four six-hourly
samples (\pm s.e.m.).

DIAGRAM 3.1

Twenty-four hour urinary thioether elimination of control, smoking (n = 14) and non-smoking (n = 11) subjects.

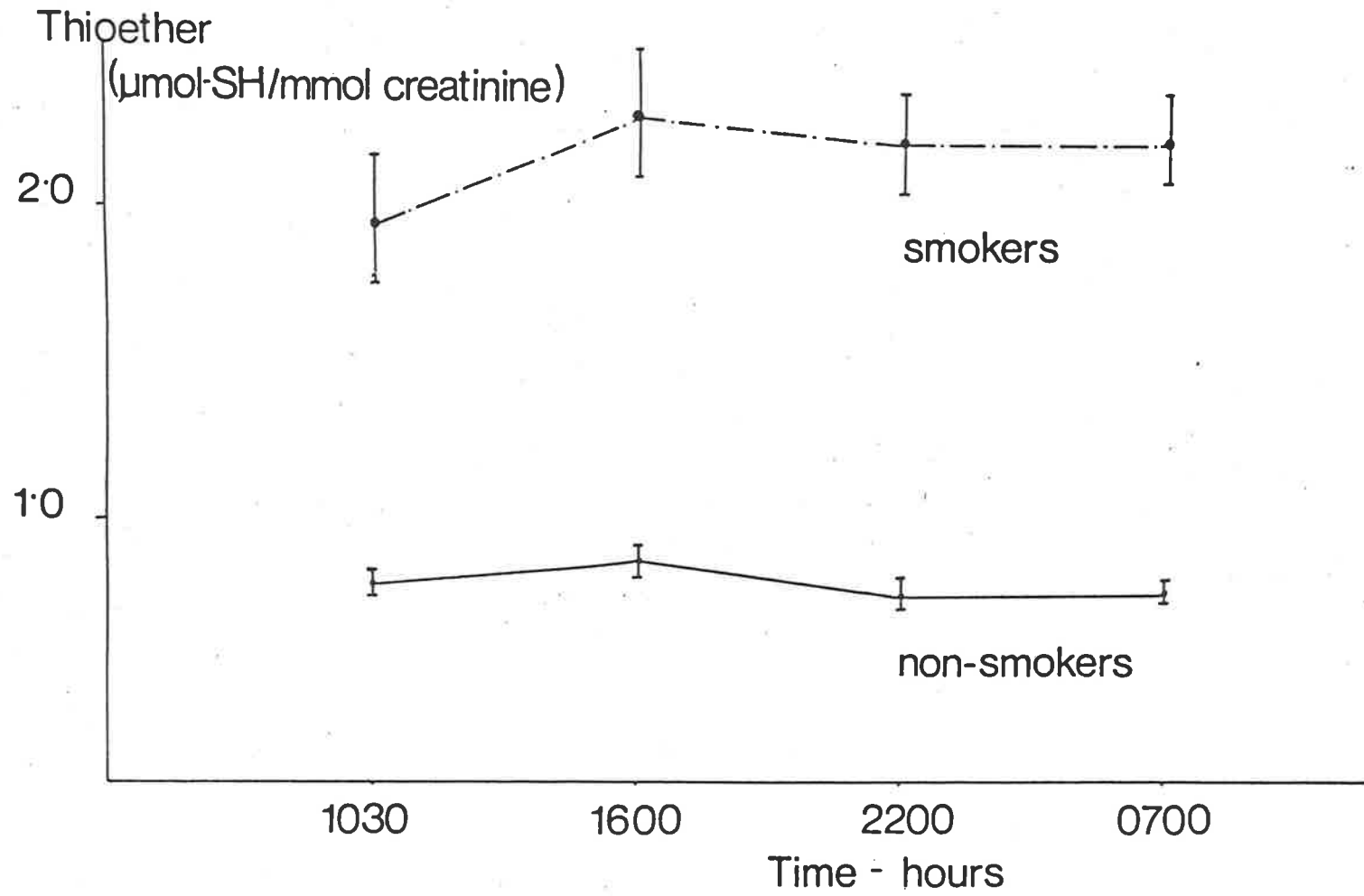


TABLE 3.2

Work-related urinary thioether output of 13 research laboratory staff.

Subject	S/NS	Thioether ($\mu\text{mol-SH}/\text{mmol creatinine}$)					
		NWD ¹			WD ¹		
		a.m.	p.m.	Δ	a.m.	p.m.	Δ
1	NS	0.73	0.63	-0.10	0.63	0.36	-0.27
2	NS	0.49	1.05	0.56	1.33	1.36	0.03
3	NS	0.37	0.40	0.03	0.30	0.63	0.33
4	S	2.05	2.69	0.64	1.57	1.96	0.39
5	S	2.61	2.61	-	2.61	3.45	0.84
6	S	0.64	0.64	-	0.64	1.73	1.09
7	NS	0.27	0.66	0.39	0.27	2.70	2.43
8	NS	0.59	0.45	-0.14	0.43	3.04	2.61
9	NS	1.32	1.39	0.07	2.97	6.23	3.26
10	NS	0.29	0.49	0.20	0.44	3.89	3.45
11	NS	0.73	1.07	0.34	1.46	6.31	4.85
12	NS	0.70	0.73	0.03	0.82	9.81	8.99
13	S	1.59	1.69	0.10	1.59	21.2	19.61

1 NWD = non-working day

WD = working day

attended petrol outlets excreted significantly higher levels of thioethers after a normal working day. (Refer to Table 3.3, Wilcoxon matched-pairs signed-ranks test, $P < 0.001$, two-tail). Work-related increases ranged from 0.64 to 80.6 $\mu\text{mol-SH}/\text{mmol creatinine}$. However, of individuals employed in self-service outlets, only smoking employees showed enhanced thioether elimination following a normal working day, ranging from 1.23 to 5.01 $\mu\text{mol-SH}/\text{mmol creatinine}$. (Refer to Table 3.4, Wilcoxon matched-pairs signed-ranks test, $P < 0.05$, two-tail). Work-associated increases in urinary thioether output were significantly greater for driveway service petrol station attendants. (Refer to Diagram 3.2, Mann-Whitney U-test, $P < 0.01$, two-tail.)

While most employees of self-service outlets were engaged in remote operation of cash register and pump controls, some also undertook mechanical workshop duties. However, in neither self-service nor attendant-operated stations was there any indication that workshop duties involving, for example, handling of lubricants, degreasers and petroleum products, and exposure to exhaust fumes within a relatively confined area, were a significant factor in increasing urinary thioether excretion. (Refer to Diagram 3.3, Chi-square analysis, $P > 0.10$.) This result therefore tends to imply that petrol is the main source of urinary thioethers excreted.

Questionnaire data on diet, alcohol intake and medications taken revealed no associations with urinary thioether excretion. Distribution of thioether data was random with respect to alcohol consumption (one-sample runs test, $P > 0.10$).

The only factor, other than driveway exposure to petroleum products, which could be identified as being associated with elevations in urinary thioether output was cigarette smoking.

TABLE 3.3

Work-related thioether excretion of 35 employees of driveway-service petrol stations.

Subject	S/NS ¹	Alcohol ² Intake	Workshop ³ Duties	Thioether ($\mu\text{mol-SH}/\text{mmol}$ creatinine)		
				a.m.	p.m.	Δ
1	NS	1	Y	2.49	2.44	-0.05
2	S	2	Y	4.36	5.00	0.64
3	S	0	N	2.01	2.89	0.88
4	NS	1	Y	1.73	2.62	0.89
5	NS	0	N	1.98	3.37	1.39
6	S	0	N	0.99	2.49	1.50
7	NS	1	Y	1.00	2.58	1.58
8	NS	0	N	2.01	3.86	1.85
9	NS	1	N	2.06	4.44	2.38
10	NS	0	Y	0.73	3.23	2.50
11	S	0	Y	1.77	4.59	2.82
12	NS	0	N	2.09	4.93	2.84
13	NS	0	N	2.00	4.91	2.91
14	NS	0	Y	0.71	3.77	3.06
15	S	1	N	3.14	6.47	3.33
16	NS	1	N	1.68	5.03	3.35
17	S	0	N	2.98	6.93	3.95
18	S	0	Y	3.39	7.55	4.16
19	NS	0	N	1.17	5.46	4.29
20	S	1	N	2.12	6.41	4.29
21	S	1	Y	1.56	6.41	4.85
22	NS	2	Y	0.94	6.46	5.52
23	S	0	N	2.12	7.92	5.80
24	NS	2	Y	3.62	10.1	6.48
25	NS	2	Y	1.63	9.10	7.47
26	S	2	N	4.35	12.0	7.65
27	NS	2	N	0.95	8.85	7.90
28	S	1	Y	3.23	12.0	8.77
29	S	0	Y	1.69	13.3	11.61
30	S	1	Y	1.95	23.8	21.85
31	S	0	Y	3.50	56.3	52.8
32	S	2	Y	1.45	58.5	57.05
33	S	2	Y	1.90	60.0	58.1
34	S	1	N	2.63	74.4	71.77
35	S	1	Y	4.38	85.0	80.62

1 S = smoker; NS = non-smoker.

2 0 = no alcohol; 1 = ≤ 1 bottle beer/day or equivalent; 2 = > 1 bottle beer/day or equivalent.

3 Y = associated workshop duties; N = no workshop duties.

TABLE 3.4

Work-related thioether output of 13 employees of self-service petrol stations.

Subject	NS/S	Alcohol Intake	Workshop Duties	Thioether ($\mu\text{mol-SH}/\text{mmol creatinine}$)		
				a.m.	p.m.	Δ
1	S	2	Y	1.82	1.59	-0.23
2	NS	2	Y	1.43	1.83	0.40
3	NS	0	N	0.65	1.13	0.48
4	NS	0	N	1.45	2.06	0.61
5	NS	0	Y	1.42	2.06	0.64
6	NS	0	N	1.06	1.79	0.73
7	NS	0	Y	0.84	2.07	1.23
8	S	1	N	1.23	2.46	1.23
9	S	2	Y	1.16	2.97	1.81
10	S	2	N	2.12	4.24	2.12
11	S	2	Y	0.93	4.14	3.21
12	NS	0	N	1.32	5.37	4.05
13	S	1	N	1.25	6.26	5.01

DIAGRAM 3.2

Work-related urinary thioether output of petrol station attendants employed in driveway (n = 35) and self-service (n = 13) stations. Values are given as mean \pm s.e.m. Smokers are represented by (□) and non-smokers by (▨).

THIOETHER
 $\mu\text{mol SH}/\text{mmol creatinine}$

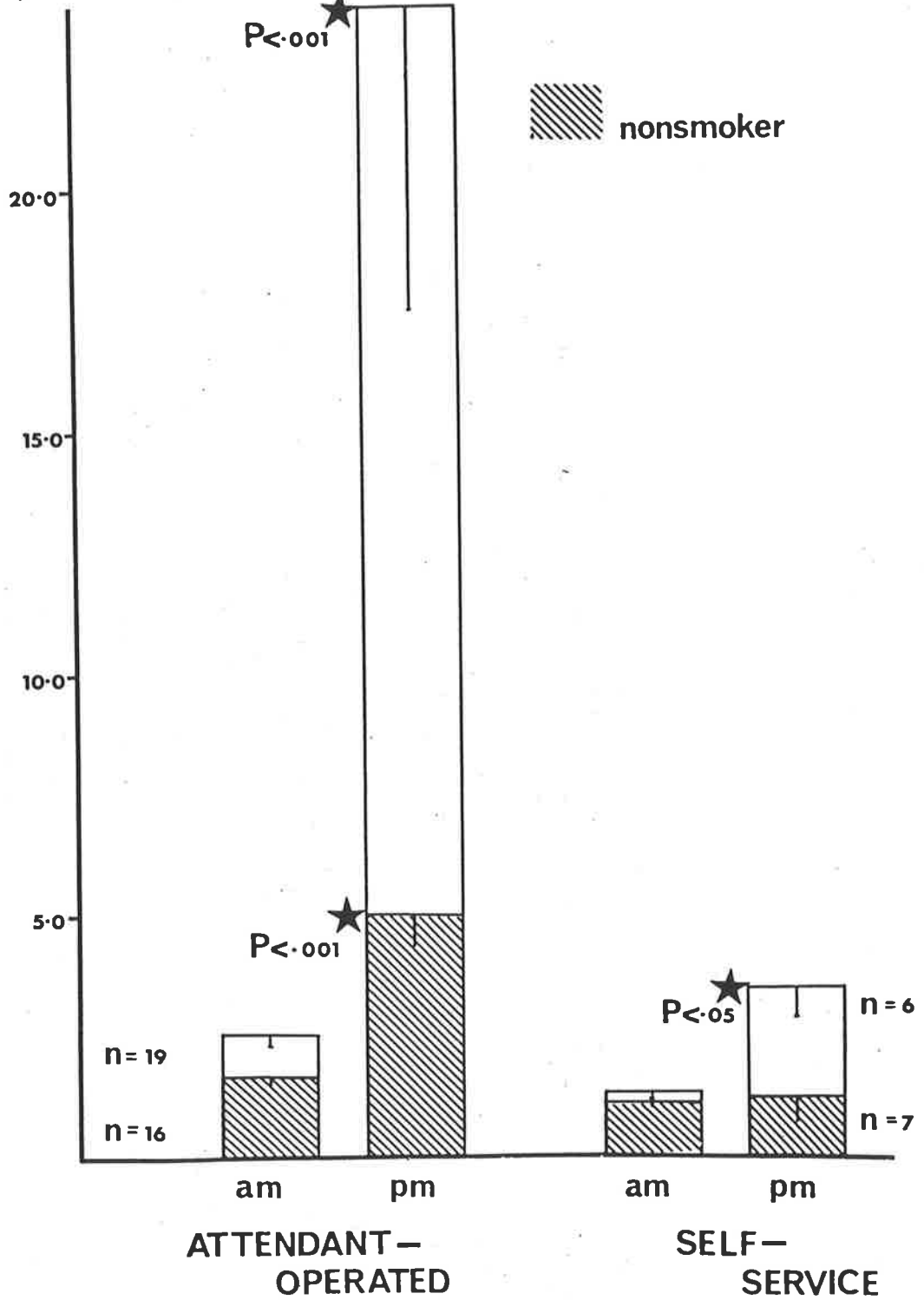


DIAGRAM 3.3

Work-related urinary thioether output of attendants employed in driveway (n = 35) and self-service (n = 13) petrol stations. Workers involved in mechanical workshop duties are represented by (O).

THIOETHER

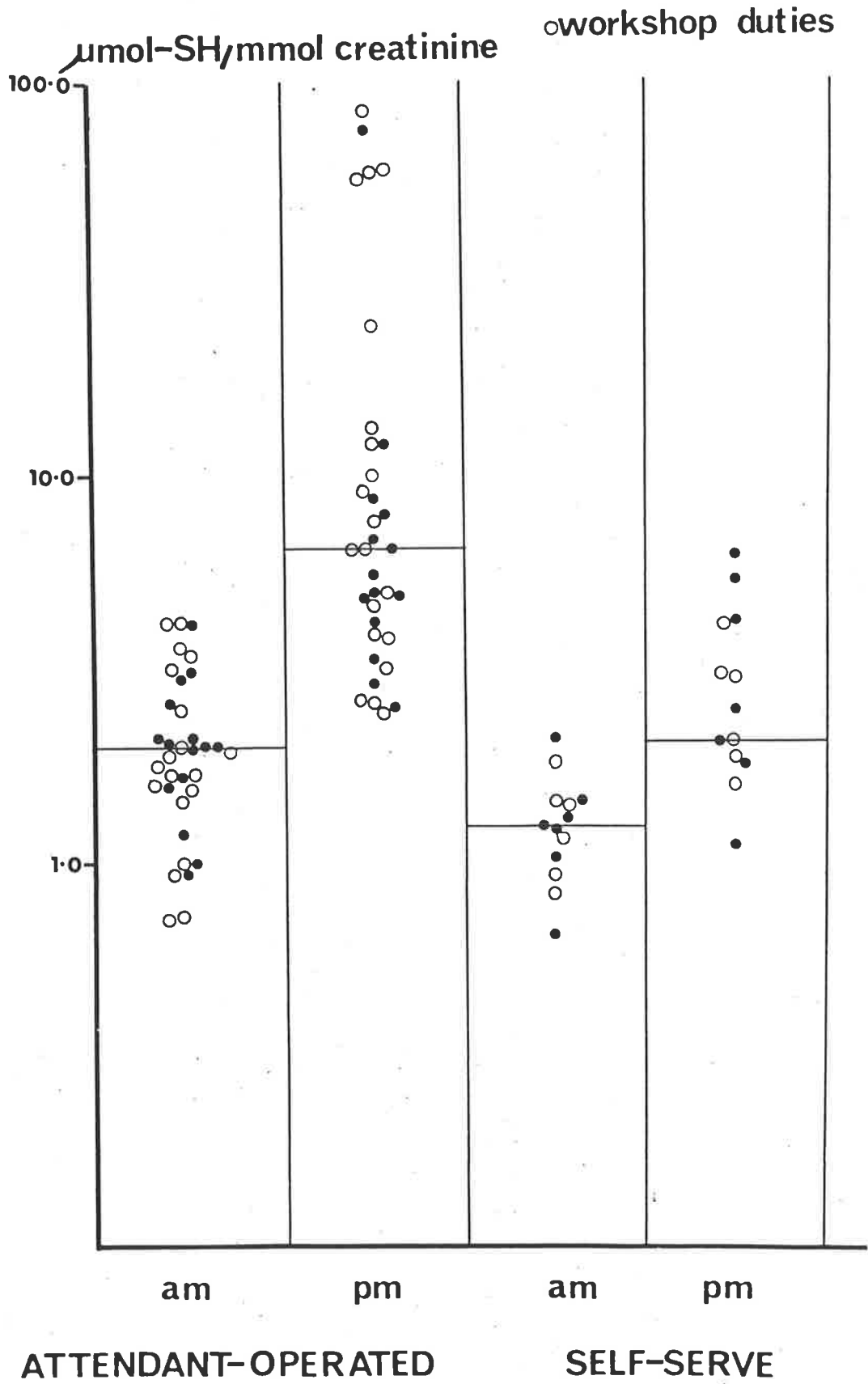


DIAGRAM 3.4

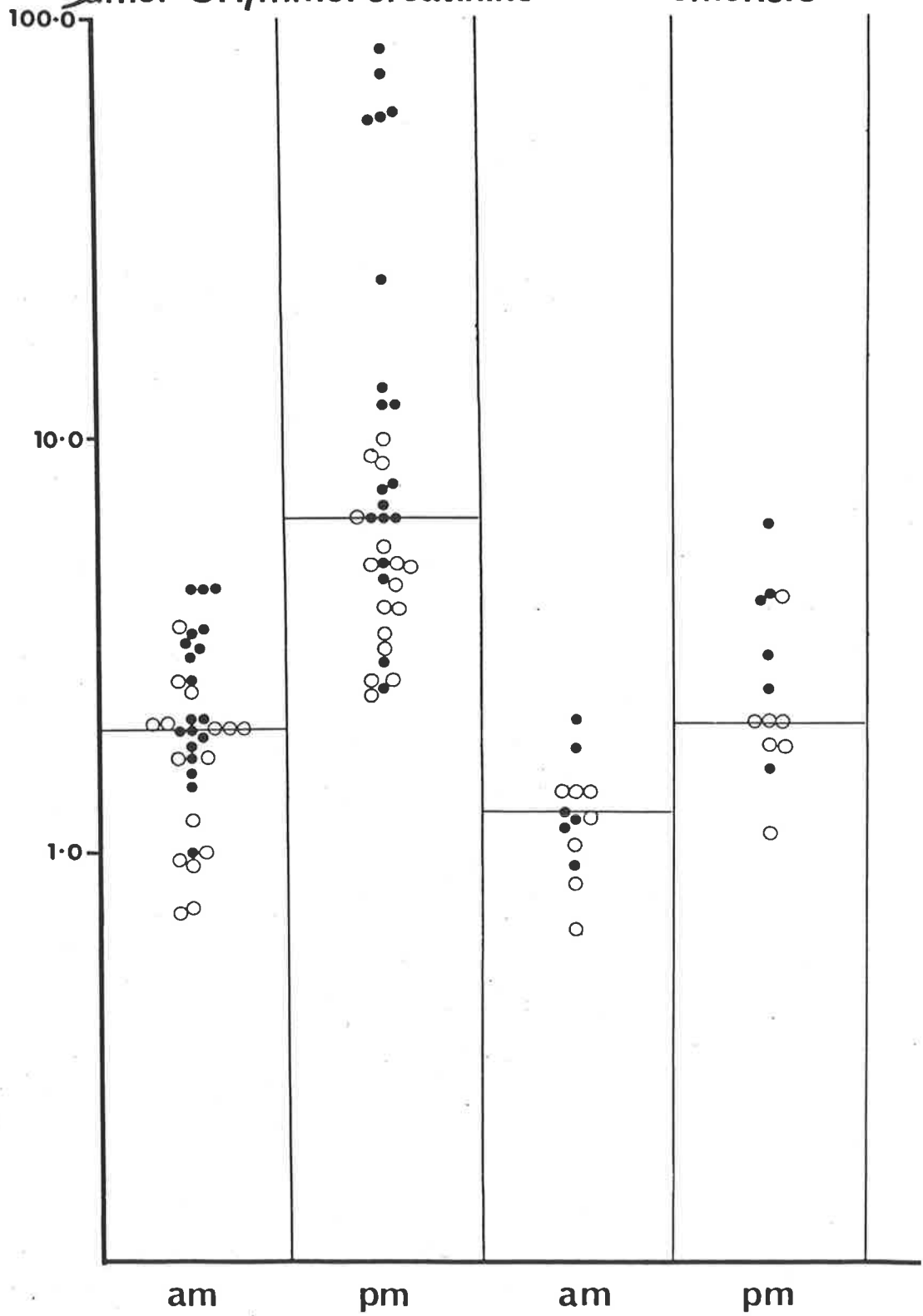
Work-related urinary thioether output of attendants employed in driveway (n = 35) and self-service (n = 13) petrol stations. Cigarette smokers are represented by (●).

THIOETHER

$\mu\text{mol-SH}/\text{mmol creatinine}$

○ non-smokers

● smokers



ATTENDANT-OPERATED

SELF-SERVE

Although not statistically significant (one-sample runs test, $P > 0.07$), there was a trend towards clustering of smokers at higher thioether excretion. (Refer to Tables 3.3, 3.4.) Cigarette smokers excreted higher levels of thioethers in both pre-work and post-work samples. (Refer to Diagram 3.4, Mann-Whitney U-test, $P < 0.001$). Furthermore, a significant interaction between smoking and work-related urinary thioether output was demonstrated (Chi-square analysis, $P < 0.01$).

Discussion

Urinary thioether output of 'control' subjects was consistently low and independent of possible diurnal variations in enzyme activity. Therefore, provided that pharmacokinetic characteristics of chemicals present in the working environment are considered, 'spot' urine sample collections at the end of the working day should indicate increased thioether excretion resulting from occupational chemical exposure.

In each group of subjects - 'control' and occupational groups - smokers excreted higher levels of urinary thioethers, consistent with reports from previous studies (van Doorn et al. 1979; van Doorn et al. 1981a). Although not distinguished within data presented, heavy smokers (refer to Table 3.1, subjects 21 to 25, all smoking greater than 20 cigarettes per day) tended to excrete higher concentrations of thioethers.

Enhancement of urinary thioether elimination produced by cigarette smoking may be explained by two different mechanisms. Elevation in thioether output may be directly attributable to bio-transformation of cigarette smoke constituents, involving clearance

via GSH conjugation. Secondly, inductive effects of cigarette smoke and components on microsomal monooxygenase activity, previously demonstrated by Abramson et al. (1977), Ali et al. (1980) and Kaur & Ali (1981), may result in stimulation of metabolism and consequent increased formation of electrophilic oxidative metabolites from various dietary components, and hence increased substrate availability for GSH conjugation. Jointly these mechanisms may explain smoking-related increases in urinary thioether output.

Work-associated elevations in urinary thioether output were generally observed for both occupational groups. However, enhancement of thioether elimination was more pronounced for driveway petrol station attendants, maximum post-work concentrations of 85.0 $\mu\text{mol-SH}/\text{mmol creatinine}$ being excreted. Increased urinary thioether excretion may be directly attributable to increased chemical exposure within the workplace. Alternatively, effects of occupational chemical exposure on microsomal monooxygenase activity may also account for increased mercapturate clearance.

A previous study by Harman et al. (1981) reported increased clearance of antipyrine, a metabolic probe drug, amongst petrol station attendants, attributable to enzyme induction by petrol vapour inhalation. This was verified by findings of increased oxidative metabolism of rats exposed to petrol vapour under similar conditions as present in the working situation. Therefore, both increased chemical exposure and stimulation of chemical biotransformation may jointly explain work-related increases in urinary thioether output.

As previously discussed in Chapter 2, due to the nonspecificity of the technique, it is not possible to identify individual chemical

components of the working environment which may account for elevations in urinary thioether output. Certainly, for organic chemical laboratory staff exposed to a wide variety of chemicals in varying intensity and frequency, identification of specific chemicals as direct sources of increased thioether output is not feasible.

However, a probable source of increase in thioether elimination may be inferred from data obtained from petrol station attendants. Observations that, firstly, elevation of thioether output is not associated with performance of workshop duties and, secondly, isolation from driveway pump operation in self-service outlets reduces the magnitude of the increase tend to implicate inhalation of petrol vapour as the main source of thioether substrates. Whether these thioethers arise directly - the petroleum components may include aliphatic and aromatic halides and alpha-beta unsaturated ketones, compounds reported by Chasseaud (1979) to commonly act as direct substrates for GSH-transferases - or, indirectly, as epoxides or other electrophilic metabolites of the microsomal mixed function monooxygenases, cannot be determined from the data. If the latter is the main source, then the apparent interactive effect of cigarette smoking can be explained by the known ability, as shown by studies by Van Cantfort & Gielen (1981), of smoking to induce microsomal monooxygenase activity.

Analysis of urinary thioether output should be viewed as a qualitative rather than quantitative monitor, functioning to 'signal' high chemical exposure levels within the workplace. The technique does possess limitations, resulting from methodological constraints and inadequate information detailing conversion of electrophilic compounds to urinary thioethers in man, estimations of such values

being based on animal studies. Comprehensive evaluation of the technique's limitations will be made in Chapter 5.

Due to the non-invasive nature of sample collection, measurements of worker exposure can be readily determined and may be used as a guide to bioavailability of chemicals involved. Extrapolation to possible health hazards associated with occupational chemical exposure are, however, somewhat tenuous.

C H A P T E R F O U R
A P P L I C A T I O N S O F T H E T H I O E T H E R T E C H N I Q U E
I N A S S E S S M E N T O F S T Y R E N E E X P O S U R E
O F M A N A N D T H E R A T

CHAPTER 4

Introduction

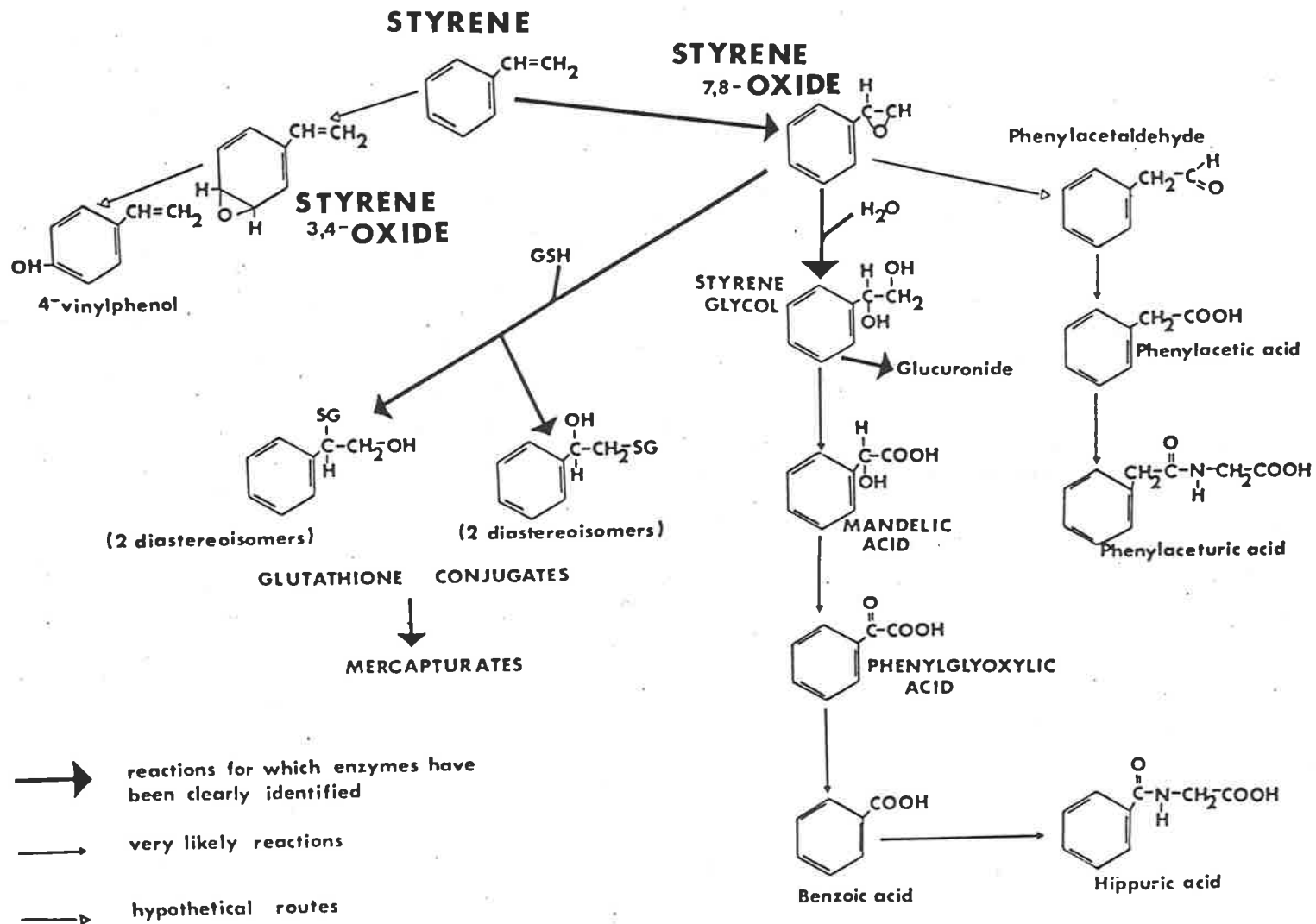
Styrene (vinylbenzene, phenylethylene) is a commercially important compound widely utilized by industry in production of polymers, co-polymers and reinforced plastics. Occupational exposure is generally greatest within reinforced plastics industries (W.H.O. Report on Styrene 1982), such as those involved in manufacture of reinforced fibreglass boats and swimming pools. Within these operations, styrene is the major air pollutant, although concomitant exposure to other compounds, such as fibrous glass, catalysts and cleaning agents, may occur. The principal routes of exposure are pulmonary and, to a lesser extent, dermal.

As shown in Diagram 4.1, the major metabolite of styrene is styrene 7,8-oxide, a reactive epoxide generated by the microsomal monooxygenases. The main detoxification mechanism involves hydration catalyzed by epoxide hydrolase (Leibman & Oritz 1969), followed by either glucuronide conjugation (Ohtsuji & Ikeda 1971) or oxidation, yielding mandelic (MA), phenylglyoxylic (PGA) and hippuric acids as subsequent urinary metabolites. Determination of urinary MA and PGA excretion has been shown to be an accurate index of styrene exposure of man in both controlled laboratory and industrial situations (Bardoděj & Bardodějova 1970; Ohtsuji & Ikeda 1970; Engström et al. 1978b; Wolff et al. 1978).

However, styrene 7,8-oxide is also a substrate for GSH-transferase, resulting in urinary elimination of mercapturic acid derivatives (Seutter-Berlage et al. 1978). Determination of urinary thioether excretion, previously shown in Chapter 3 to be increased

DIAGRAM 4.1

Metabolic pathways of styrene.



by occupational chemical exposure - particularly amongst driveway petrol station attendants - may therefore offer a simple and rapid means of monitoring chemical exposure of workers of reinforced plastics industries. Simultaneous assessment of specific styrene metabolites, MA and PGA, may provide support for styrene being the major source of chemical exposure within the working environment, and hence largely responsible for possible work-related influences on thioether elimination.

Uptake and distribution of styrene within the body is rapid. In animal studies performed by Danishefsky & Willhite (1954) and Sauerhoff & Braun (1976), the majority of a styrene dose (85%) was eliminated within 24 hours principally as urinary metabolites. Metabolite excretion appears to involve, at least, a two compartment model. However, studies by Wolff et al. (1977) and Engström et al. (1978a) have indicated the potential for accumulation of styrene in adipose tissue of fibreglass workers subjected to repeated daily exposure. Measurement of styrene sequestration in tissue stores has been suggested as a monitoring approach, but, to date, has not been adopted within industry due to practical and ethical constraints inherent to sampling strategies.

A study of chemical waste operators by van Doorn et al. (1981a) over a working week showed small increases in urinary thioether concentrations excreted in pre-work samples of successive working days, suggesting possible cumulative effects on thioether output. These results tended to indicate that urinary thioether determination may therefore provide an estimate of chemical(s) or metabolite(s) levels present prior to commencement of each working day, particularly relevant in working situations involving exposure to chemicals with

long biological half-lives. In an attempt, therefore, to assess whether metabolites, possible precursors of urinary thioethers, accumulated during the course of a working week, urinary thioether, MA and PGA excretion was investigated in follow-up studies of fibre-glass workers over a consecutive eight-day period.

Since occupational chemical exposure of workers cannot be controlled by the researcher, a model in which dosage and metabolic activity could be manipulated was necessary to investigate factors influencing styrene-related thioether output. As similar pathways of styrene metabolism have been implicated in both rats and man, the rat was chosen as an animal model - as in Chapter 2 - and adapted to studying modifying influences affecting styrene-related thioether output.

Metabolism of styrene is cytochrome P-450 dependent, at least in its primary steps, and may be potentially induced by chemicals known to stimulate microsomal monooxygenase activity. A study by Ohtsuji & Ikeda (1971), in which rats were dosed with styrene following phenobarbital pretreatment, demonstrated enhanced biotransformation of styrene to styrene 7,8-oxide with resultant increases in urinary MA and PGA excretion. Treatments with inducers relatively selective for cytochrome P-448-dependent enzymes, described in Chapter 2, may offer a comparable model for assessing and interpreting effects of cigarette smoking, previously shown to stimulate microsomal monooxygenase activity (Van Cantfort & Gielen 1981), on work-related urinary thioether output of fibreglass workers.

Most workers participating in this study had been employed within fibreglass reinforced plastics industries for a considerable period. A previous study by Vainio et al. (1979) indicated that chronic

exposure to styrene vapour (300 ppm) resulted in increased metabolic clearance of the chemical and therefore a reduction in styrene body-burden with continued long-term exposure (Savolainen & Vainio 1977). Auto-induction, suggested by these two studies, may significantly modify biological parameters of exposure and possibly compromise the validity and reliability of exposure tests. Therefore, to evaluate the significance of this effect, the rat was used as an animal model to monitor urinary thioether, MA and PGA elimination at specific time intervals during, and on completion of, chronic styrene treatment regimes.

Methods

1. Human Studies

(1) Subjects

Sixteen male and four female employees of four different fibreglass manufacturing companies (fibreglass reinforced plastic (FRP) boat construction, swimming pool construction) participated in the study. Subjects either worked in one particular area of boat/pool construction - for example, as a fibreglass laminator - or were involved in a range of duties including laminating, moulding and product assembly - for example, production foreman. All workers had been employed within fibreglass industries for more than one year and were not taking any medications at the time of study. Ages ranged from 19-62 years (34 ± 11 , mean \pm SD). Eight of the subjects participated in follow-up studies carried out six months after completion of the preliminary study.

(2) Sampling protocol

Participants in the initial study were asked to collect two urine samples on both non-working (Sunday) and working (Monday) days. Morning samples were collected between 0700 and 0800 hours and evening samples between 1900 and 2000 hours. Subjects involved in follow-up studies were asked to continue this sampling protocol for a consecutive eight-day period (incorporating five working and three non-working days). Urine samples were frozen after collection and stored frozen until assayed.

Subjects completed a short questionnaire, previously described in Chapter 3 and Appendix 4, at the time of sample collection.

2. Animal Studies

(1) Animals

Adult male Porton rats, weighing 180-220 g, were obtained from the Waite Agricultural Research Institute, South Australia, and housed in the Adelaide University Central Animal House. Untreated animals were kept in a 12-hour light/dark cycle and allowed food (standard laboratory chow) and water ad libitum.

(2) Styrene administration

(a) Acute studies

Styrene was administered in peanut oil as a single i.p. dose. Dose range given was 0.43 to 2.80 mmol/kg. Time of administration was usually 0830 hours to avoid modification of drug metabolism due to diurnal variations in microsomal enzyme activity (Tredger & Chhabra 1977). Following styrene administration, rats were housed in individual stainless steel metabolism cages, with free

access to food and water. Urine was collected daily and stored frozen until assayed.

(b) Chronic studies

Styrene 1.44 mmol/kg or 2.40 mmol/kg was administered in peanut oil as a single daily dose, i.p., five times per week, for three consecutive weeks. Urine samples were collected 24 hours following administration of styrene after 0, 1, 2 and 3 weeks of treatment. Urine samples were stored frozen until assayed.

(3) Pretreatment regimes

The following inductive pretreatments were given:

(a) Phenobarbital (PB) administered in drinking water at a concentration of 1 mg/ml for a period of 7-10 days.

(b) Beta-naphthoflavone (BNF) single i.p. dose of 200 mg/kg 40 hours prior to drug administration.

(c) Polychlorinated biphenyl (PCB) single i.p. dose of 500 mg/kg 4-5 days prior to drug administration.

Following pretreatment of animals, styrene doses, as given in Table 4.5, were administered and 24-hour urine samples collected.

3. Sample Analysis

(1) Urinary thioether and creatinine

Urinary thioether and creatinine concentrations were determined as previously described in Chapter 2.

(2) Urinary mandelic and phenylglyoxylic acid

Urinary mandelic (MA) and phenylglyoxylic acid (PGA) concentrations were determined by the method of Ohtsuji & Ikeda (1970).

Aliquots (0.5 ml) of acidified urine were extracted with 5.0 ml of ether and organic layer separated by centrifugation at 1000xg for 5 minutes. Ether extracts were collected and evaporated to dryness at 70°C; 4 ml of sulphuric acid/formalin reagent (100:1) were added to residues, and after mixing, tubes were allowed to stand 30 minutes to allow for colour development.

Concentrations of PGA and MA were determined colourimetrically using a Pye Unicam SP 1800 spectrophotometer set at 350 and 450 nm, respectively. Standard curves of both PGA and MA were prepared using urine aliquots spiked with known amounts of either acid and carried through the assay procedure. (Refer to Appendix 5, 6.)

Concentrations of both MA and PGA were expressed relative to creatinine concentrations to minimize any variations due to fluctuations in urine output.

4. Data Analysis

Non-parametric statistical analyses were employed to avoid possible erroneous assumptions regarding distribution of data.

Both Kruskal-Wallis one-way analysis of variance and Mann-Whitney U-tests for unrelated samples were used to analyze drug treatment effects on thioether, MA and PGA excretion of rats.

Differences in thioether excretion of workers due to chemical exposure or cigarette consumption were determined using the Wilcoxon matched-pairs signed-rank test and Mann-Whitney U-test, respectively. Possible effects of alcohol consumption or nature of employment duties on thioether excretion were evaluated using the one-sample runs test and Mann-Whitney U-tests, respectively. The significance of relationships between thioether and MA, thioether and PGA, and thioether and

combined MA and PGA elimination was investigated using Spearman rank correlation coefficients. Chi-square analysis was used for assessing possible interactive effects between cigarette consumption and occupational chemical exposure of man (Siegel 1956).

Results

1. Human Studies

(1) Preliminary study

(a) Urinary thioether excretion

Analysis of data presented in Table 4.1 showed that there was no statistically significant difference between urinary thioether concentrations excreted in non-working and morning working day samples (Wilcoxon matched-pairs signed-rank test, two-tail, $P > 0.10$). Smokers eliminated significantly higher levels of thioethers (ranging from 0.71 to 3.18 $\mu\text{mol-SH}/\text{mmol}$ creatinine, median 1.83 $\mu\text{mol-SH}/\text{mmol}$ creatinine) than non-smokers (range of 0.33 to 2.95 $\mu\text{mol-SH}/\text{mmol}$ creatinine, median 1.30 $\mu\text{mol-SH}/\text{mmol}$ creatinine) (Mann-Whitney U-test, two-tail, $P < 0.05$). Values measured were consistent with reported thioether output of a 'non-exposed' population. (Refer to Chapter 3, van Doorn et al. 1979.)

Significant enhancement of urinary thioether output after an eight-hour working day was observed for both smoking and non-smoking fibreglass workers. (Refer to Diagram 4.2, Wilcoxon matched-pairs signed-rank test, two-tail, $P < 0.001$). However, thioether output of smokers (range of 2.31 to 574 $\mu\text{mol-SH}/\text{mmol}$ creatinine, median of 16.1 $\mu\text{mol-SH}/\text{mmol}$ creatinine) was not statistically different from that of non-smokers (range of 0.93 to 48.3 $\mu\text{mol-SH}/\text{mmol}$ creatinine,

TABLE 4.1

Urinary thioether excretion of fibreglass workers. I. Preliminary Study.

Subject	NS/S	Work ¹ Duties	Company	Thioether ($\mu\text{mol-SH}/\text{mmol creatinine}$)					
				NWD			WD		
				a.m.	p.m.	Δ	a.m.	p.m. ²	Δ
1	S	b	A	2.29	2.08	-0.21	2.38	7.23	4.85
2	NS	a	A	1.83	1.48	-0.35	1.59	7.30	5.71
3	NS	c	B	0.53	0.44	-0.09	0.34	6.02	5.68
4	S	c	B	1.01	1.46	0.45	0.71	7.76	7.05
5	NS	d	B	1.19	1.40	0.21	1.43	11.8	10.37
6	S	b	B	1.60	1.42	-0.18	1.70	26.7	25.0
7	NS	c	B	2.68	2.95	0.27	2.66	44.3	41.64
8	NS	a	B	1.54	2.17	0.63	1.06	48.3	47.24
9	S	a	B	2.56	1.05	-1.51	3.16	574 *	570.8
10	S	c	C	1.40	2.35	0.95	1.92	2.31	0.39
11	S	c	C	3.18	3.04	-0.14	3.01	4.72	1.71
12	NS	a	C	1.43	1.72	0.29	1.04	13.9	12.86
13	S	b	C	1.61	1.61	-	2.05	27.3	25.25
14	S	d	C	1.68	1.74	0.06	1.91	24.5	22.59
15	S	a	C	1.74	2.62	0.88	2.06	81.8	79.74
16	NS	e	D	0.63	0.33	-0.30	0.37	0.93	0.56
17	NS	c	D	0.56	0.80	0.24	0.85	1.44	0.59
18	NS	b	D	0.68	0.97	0.29	0.62	2.52	1.90
19	NS	c	D	1.59	1.81	0.22	1.79	3.71	1.92
20	S	c	D	2.09	1.85	-0.24	1.82	3.88	2.06

1 Work duties: a: laminator; b: moulder; c: assembly; d: production foreman; e: storeman.

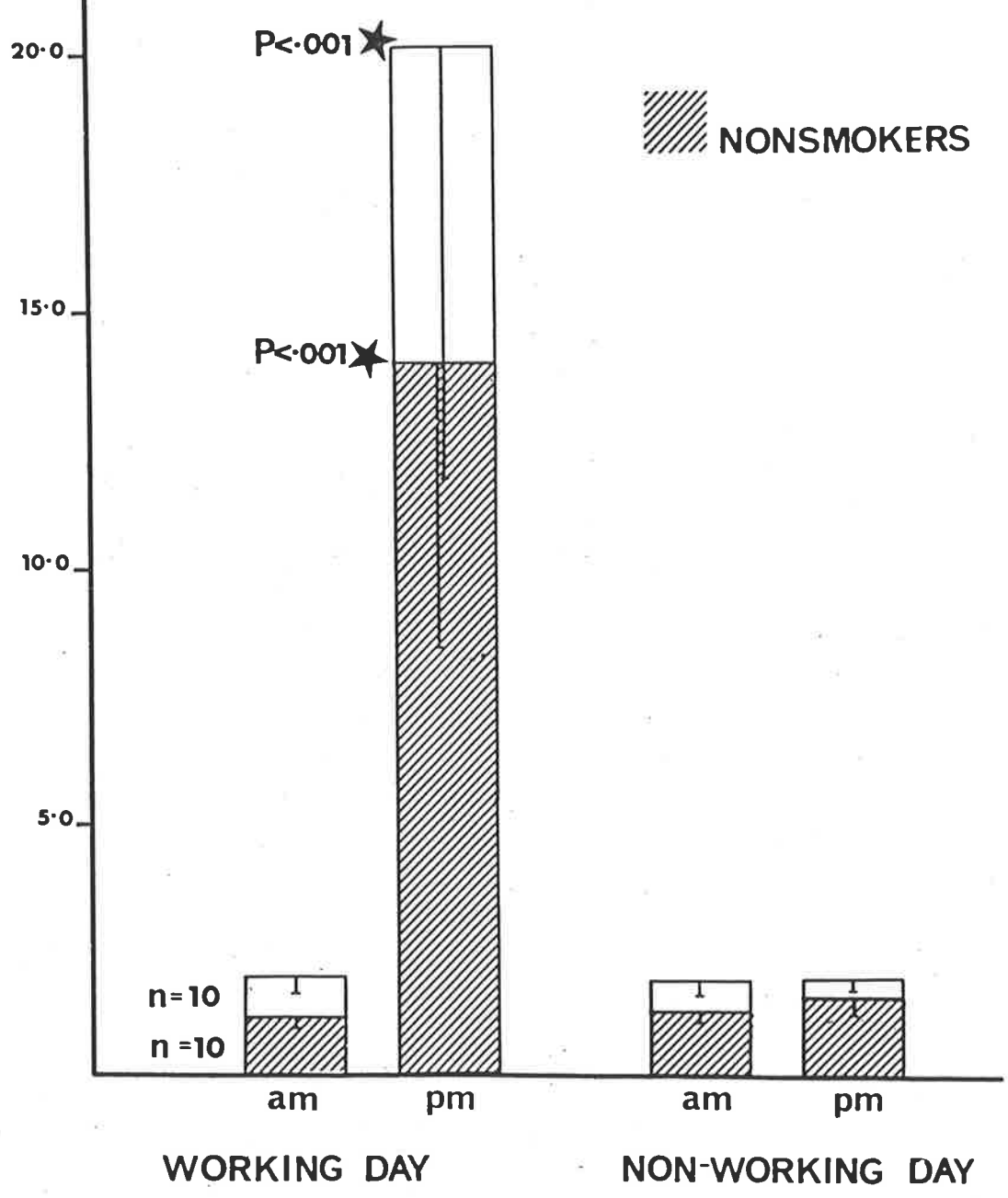
2 Significantly different from pre-work thioether excretion, $P < 0.001$ (Wilcoxon matched-pairs signed rank test, two-tail).

* Individual involved in fibreglass spraying all day.

DIAGRAM 4.2

Urinary thioether elimination of 20 fibreglass workers after both non-working (Sunday) and eight-hour working (Monday) days. Values are given as mean \pm s.e.m., smokers (n = 10) (\square), non-smokers (n = 10) ($\text{\textcircled{/}}$).

THIOETHER
umol/mmol creatinine



and median of 6.76 $\mu\text{mol-SH}/\text{mmol creatinine}$) (Mann-Whitney U-test, two-tail, $P>0.10$). This result may be explained by marked variations of ambient concentrations of occupational chemicals within each factory associated with different stages of product manufacture.

Individuals employed as fibreglass laminators excreted markedly higher levels of thioethers (range of 7.30 to 574 $\mu\text{mol-SH}/\text{mmol creatinine}$ with median of 65 $\mu\text{mol-SH}/\text{mmol creatinine}$) than workers involved in product assembly (range of 1.44 to 13.9 $\mu\text{mol-SH}/\text{mmol creatinine}$ and median of 3.88 $\mu\text{mol-SH}/\text{mmol creatinine}$) (Mann-Whitney U-test, two-tail, $P<0.02$). Further evidence of variations in ambient chemical concentrations within the workplace is provided by a very high thioether output of 574 $\mu\text{mol-SH}/\text{mmol creatinine}$ excreted by one fibreglass laminator continuously involved in fibreglass spraying during an eight-hour working day.

Generally, workers of Company B tended to excrete higher thioether levels than employees of other companies, possibly attributable to poor ventilation systems, particularly in areas of fibreglass sprayers, for removal of styrene vapours and other environmental pollutants.

Alcohol consumption had no effect on work-related thioether output (one-sample runs test, $P>0.10$). Chi-square contingency analysis revealed no significant interactive effects between cigarette consumption and occupational chemical exposure on urinary thioether excretion as had been previously demonstrated for petrol station attendants. (Refer to Chapter 3.)

(b) Urinary MA and PGA excretion

Urinary concentrations of MA and PGA eliminated by employees following a normal working day, presented in Table 4.2, were consistent

TABLE 4.2

Work-related urinary thioether, mandelic acid (MA) and phenylglyoxylic acid (PGA) of fibreglass employees. I. Preliminary Study.

Subject	NS/S	Work Duties	Company	WD Thioether ($\mu\text{mol-SH}/\text{mmol creatinine}$)			MA ¹	PGA ²	MA+PGA ³
				a.m.	p.m.	Δ	(mmol/mmol creatinine)		
1	S	b	A	2.38	7.23	4.85	0.30	0.11	0.41
2	NS	a	A	1.59	7.30	5.71	0.46	0.15	0.61
3	NS	c	B	0.34	6.02	5.68	0.70	0.15	0.85
4	S	c	B	0.71	7.76	7.05	1.83	0.26	2.09
5	NS	d	B	1.43	11.8	10.37	0.82	0.10	0.92
6	S	b	B	1.70	26.7	25.0	2.03	0.14	2.17
7	NS	c	B	2.66	44.3	41.64	1.83	0.26	2.09
8	NS	a	B	1.06	48.3	47.24	2.30	1.78	4.08
9	S	a	B	3.16	574	570.8	10.0	8.20	18.2
10	S	c	C	1.92	2.31	0.39	0.10	0.06	0.16
11	S	c	C	3.01	4.72	1.71	0.74	0.24	0.98
12	NS	a	C	1.04	13.9	12.86	1.16	0.55	1.71
13	S	b	C	2.05	27.3	25.25	1.76	1.16	2.92
14	S	d	C	1.91	24.5	22.59	0.58	0.25	0.83
15	S	a	C	2.06	81.8	79.74	5.37	3.67	9.04
16	NS	e	D	0.37	0.93	0.56	0.22	0.08	0.30
17	NS	c	D	0.85	1.44	0.59	0.47	0.15	0.62
18	NS	b	D	0.62	2.52	1.90	0.72	0.41	1.13
19	NS	c	D	1.79	3.71	1.92	0.40	0.34	0.74
20	S	c	D	1.82	3.88	2.06	1.09	0.47	1.56

- 1 Thioether vs MA excretion, $r_s = 0.82$, $P < 0.01$.
- 2 Thioether vs PGA excretion, $r_s = 0.59$, $P < 0.05$.
- 3 Thioether vs (MA+PGA) excretion, $r_s = 0.80$, $P < 0.01$.

with values previously reported by Guillemin & Bauer (1979) and Ikeda et al. (1982). Significant rank correlations were shown between

- (a) urinary thioether and MA excretion - $r_s = 0.82$, $P < 0.005$;
- (b) urinary thioether and PGA excretion - $r_s = 0.59$, $P < 0.05$;
- (c) urinary thioether and combined MA and PGA excretion - $r_s = 0.80$, $P < 0.005$.

(2) Follow-up studies

Urinary thioether, MA and PGA levels excreted by fibreglass workers over a consecutive eight-day period are presented in Diagrams 4.3 and 4.4, and summarized in Table 4.3.

Individuals employed in Company B excreted markedly lower levels of thioethers on the corresponding working day (Monday) than values determined in the preliminary study. Urinary thioether output of workers of Company D determined on working day 1 (Monday) was consistent with values reported in the preliminary study. (Refer to Table 4.4, Wilcoxon matched-pairs signed-rank test, two-tail, $P > 0.10$). Excluding subject 20, values determined for workers were within the range of concentrations excreted by a 'non-exposed' population. (Refer to Chapter 3, van Doorn et al. 1979).

Workers of Company D showed a trend for elevation of urinary thioether output with progression of the working week. Increases produced could not be explained by cumulative effects of chemical exposure, as thioether concentrations measured in pre-work samples for each individual were uniformly low and within the range of values excreted by a 'non-exposed' population. (Refer to Chapter 3.)

Significant rank correlations between

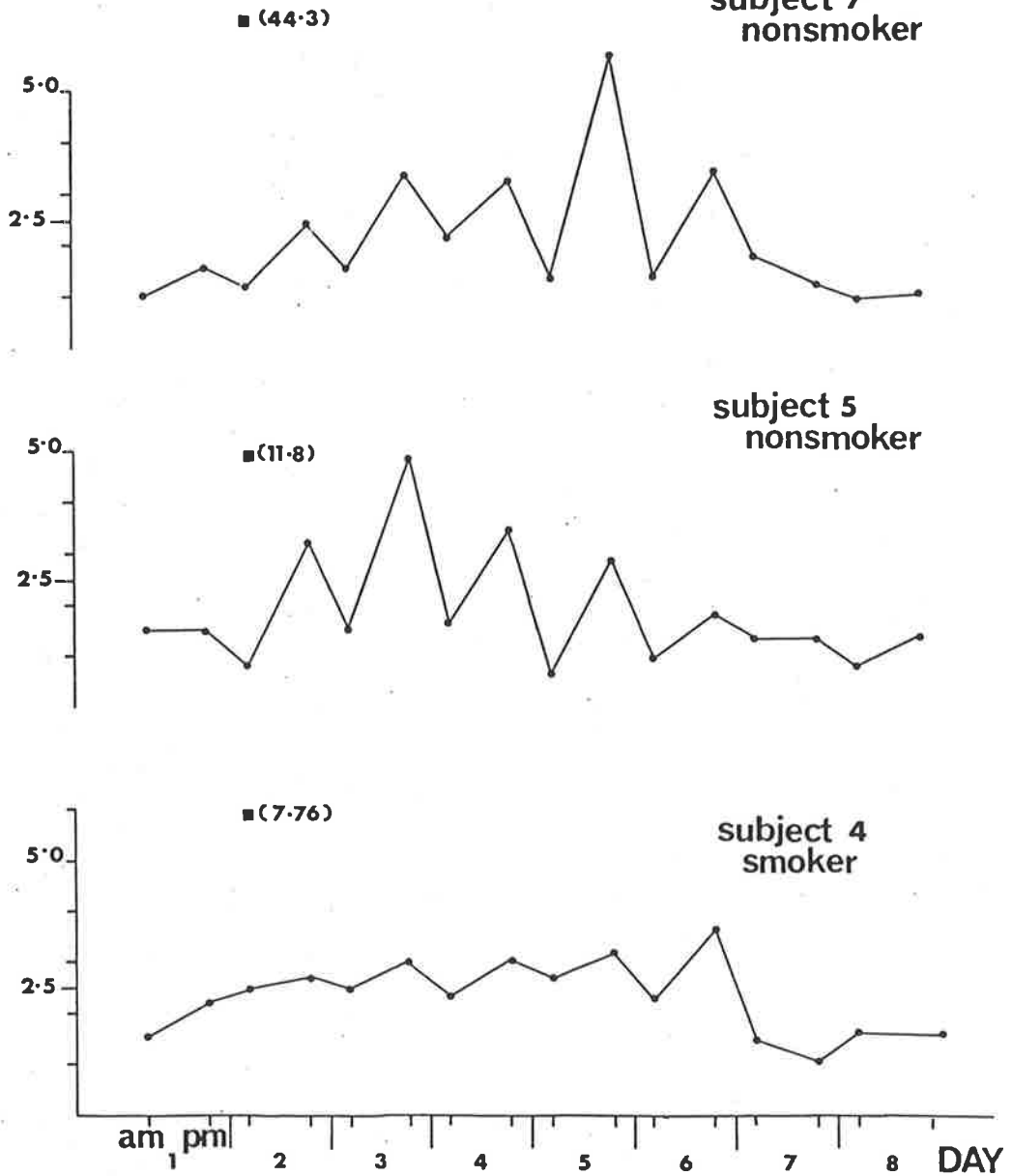
- (a) urinary thioether and MA excretion - $r_s = 0.82$, $P < 0.005$;
- (b) urinary thioether and PGA excretion - $r_s = 0.59$, $P < 0.05$;

DIAGRAM 4.3

Concentrations of urinary thioethers excreted by 3 employees of Company B over a consecutive eight-day period. Work-related urinary thioether output measured in the preliminary study is included for comparison.

COMPANY B

THIOETHER
 $\mu\text{mol-SH}/\text{mmol creatinine}$



■ (work-related thioether output-preliminary study)

DIAGRAM 4.4

Concentrations of urinary thioethers excreted by 5 employees of Company D over a consecutive eight-day period. Work-related urinary thioether output measured in the preliminary study is included for comparison.

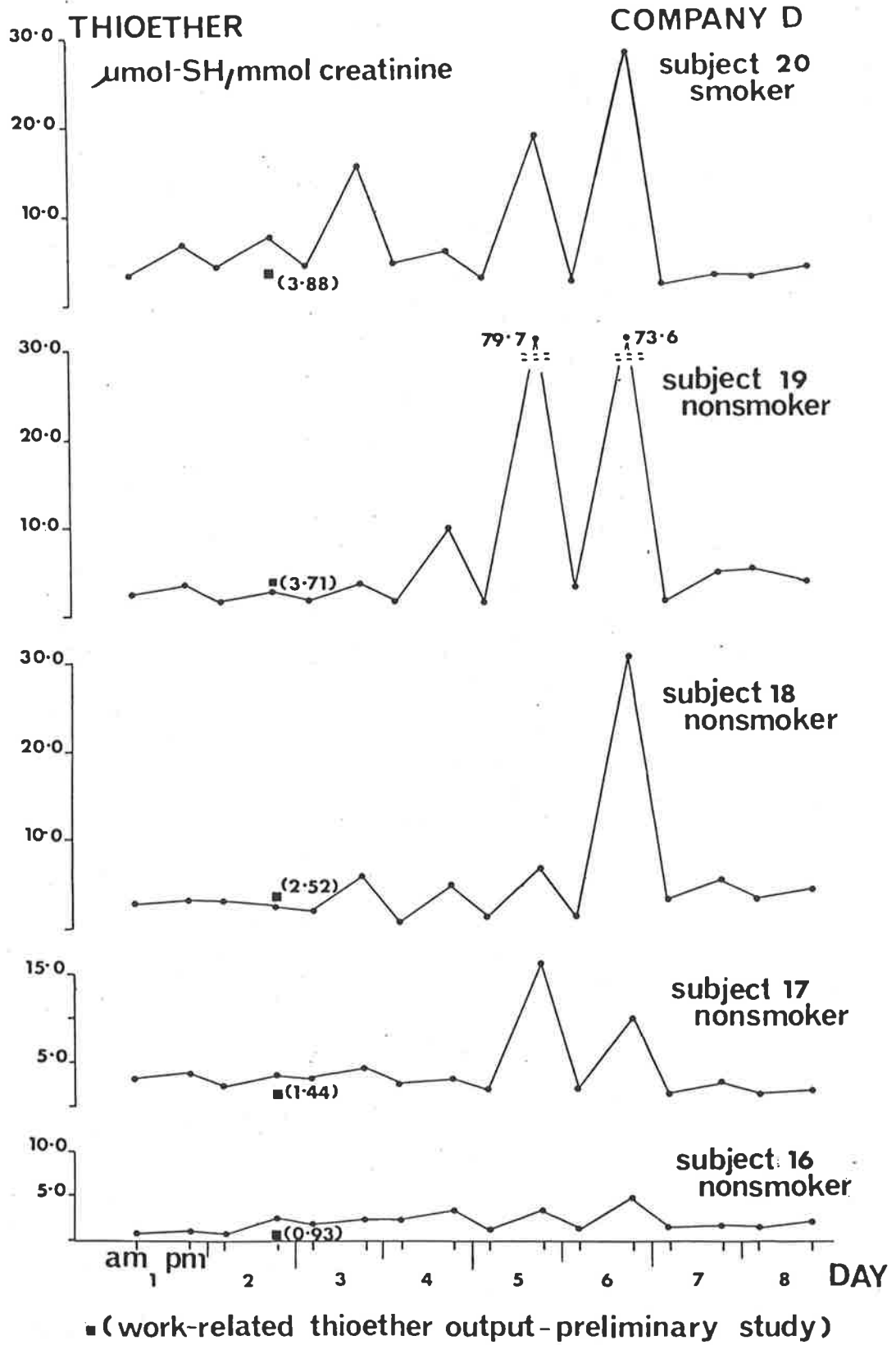


TABLE 4.3

Urinary thioether, mandelic acid (MA) and phenylglyoxylic acid (PGA) excretion of fibreglass workers. II. Follow-up Study.

Day (W/NW)	Thioether ($\mu\text{mol-SH}/\text{mmol creatinine}$)		MA ¹	PGA ²	(MA+PGA) ³
	a.m.	p.m.			
(a) Smokers (n = 4)					
1 NW	3.01 (0.17)	4.70 (0.92)	-	-	-
2 W	2.69 (0.45)	4.07 (1.08)	0.16 (0.02)	0.05 (0.01)	0.21 (0.03)
3 W	3.33 (0.85)	6.61 (2.97)	0.30 (0.06)	0.08 (0.02)	0.37 (0.08)
4 W	2.10 (0.85)	5.07 (1.95)	0.34 (0.09)	0.07 (0.02)	0.41 (0.10)
5 W	2.18 (0.34)	23.8 (14.4)	0.66 (0.29)	0.17 (0.07)	0.83 (0.36)
6 W	2.43 (0.44)	23.7 (13.2)	0.73 (0.30)	0.19 (0.08)	1.05 (0.32)
7 NW	1.72 (0.23)	3.01 (0.61)	-	-	-
8 NW	2.19 (0.71)	2.04 (0.51)	-	-	-
(b) Non-Smokers (n = 4)					
1 NW	1.55 (0.62)	1.85 (0.47)	-	-	-
2 W	1.50 (0.53)	2.69 (0.20)	0.12 (0.02)	0.05 (0.01)	0.17 (0.02)
3 W	2.25 (0.45)	4.27 (0.61)	0.18 (0.04)	0.06 (0.02)	0.24 (0.05)
4 W	1.75 (0.31)	3.66 (0.72)	0.16 (0.03)	0.06 (0.02)	0.21 (0.04)
5 W	1.09 (0.09)	4.65 (0.91)	0.27 (0.11)	0.08 (0.03)	0.35 (0.14)
6 W	1.19 (0.09)	10.0 (6.73)	0.51 (0.25)	0.14 (0.08)	0.67 (0.40)
7 NW	1.92 (0.39)	2.39 (0.83)	-	-	-
8 NW	1.41 (0.62)	2.03 (0.74)	-	-	-

Thioether, MA and PGA are given as mean (\pm s.e.m.), n = 4.

1 Thioether vs MA excretion, $r_s = 0.82$, $P < 0.01$.

2 Thioether vs PGA excretion, $r_s = 0.59$, $P < 0.05$.

3 Thioether vs (MA+PGA) excretion, $r_s = 0.80$, $P < 0.01$.

TABLE 4.4

Comparison of urinary thioether excretion by 8 fibreglass workers measured after working day 1 (Monday) of preliminary and follow-up studies.

Subject	Company	Thioether ($\mu\text{mol-SH}/\text{mmol creatinine}$)		
		Preliminary	Follow-up	Δ
4	B	7.76	2.73	-5.03
5	B	11.8	3.25	-8.55
7	B	44.3	2.38	-41.92
16	D	0.93	2.63	1.70
17	D	1.44	3.49	2.05
18	D	2.52	2.49	-0.03
19	D	3.71	2.79	-0.92
20	D	3.88	7.27	3.39

(c) urinary thioether and combined MA and PGA excretion -

$$r_s = 0.80, P < 0.005$$

were demonstrated, consistent with values reported in the preliminary study.

2. Animal Studies

(1) Acute studies

(a) Effect of styrene

Administration of styrene (0.43-2.80 mmol/kg) produced significant dose-related increases in urinary thioether output (Mann-Whitney U-test, two tail; refer to Table 4.5 for values and significance levels). A maximum increase of 290 $\mu\text{mol-SH}/\text{mmol creatinine}$ was determined following administration of 2.80 mmol/kg, representing an increase of 660-fold above control thioether output.

Significant Spearman rank order correlations between styrene-related thioether excretion and MA, PGA and combined MA and PGA elimination were shown ($r_s = 0.74, 0.73$ and 0.77 , respectively, all $P < 0.001$).

(b) Effect of pretreatments

Induction of microsomal enzyme activity by pretreatment with both cytochrome P-448-specific and general enzyme inducers significantly increased styrene-related thioether, MA and PGA elimination. (Refer to Diagram 4.5, Mann-Whitney U-test, two-tail; refer to Table 4.5 for significance levels). All pretreatments produced comparable enhancement of MA and PGA elimination of approximately 2 to 3-fold. However, after accounting for the effects of inductive pretreatments on baseline-thioether output (refer to Chapter 2), BNF was shown to produce the greatest magnitude of styrene-related thioether increase.

TABLE 4.5

Urinary thioether, mandelic acid (MA) and phenylglyoxylic acid (PGA) elimination of both control and treated rats 24 hours following administration of styrene. I. Acute Styrene Treatment.

Treatment	Dose (mmol/kg)	Thioether ($\mu\text{mol-SH}/\text{mmol creatinine}$)	MA ¹	PGA ² (mmol/mmol creatinine)	(MA+PGA) ³
Control	-	0.44 (0.01)*	-	-	-
	0.43	48.6 (7.72)	0.28 (0.05)	0.06 (0.01)	0.34 (0.06)
	0.86	89.2 (10.2)	0.43 (0.17)	0.06 (0.01)	0.49 (0.17)
	1.44	140 (18.7)	0.52 (0.12)	0.14 (0.04)	0.66 (0.14)
	1.92	189 (18.2)	0.84 (0.18)	0.31 (0.09)	1.15 (0.25)
	2.40	219 (8.53)	1.09 (0.31)	0.48 (0.06)	1.61 (0.23)
	2.80	290 (20.9)	1.23 (0.24)	0.58 (0.05)	1.80 (0.35)
PB	0.43	82.3 (9.35) ^a	0.60 (0.02) ^b	0.12 (0.01) ^a	0.72 (0.02) ^d
1 mg/ml in drinking water, 7-10 days	1.44	254 (40.7) ^c	1.50 (0.02) ^d	0.36 (0.02) ^c	1.86 (0.07) ^d
	2.40	485 (29.3) ^d	2.65 (0.13) ^b	0.84 (0.05) ^a	3.49 (0.15) ^d
PCB	0.43	69.8 (4.12) ^a	0.54 (0.16)	0.17 (0.04) ^a	0.71 (0.16) ^a
500 mg/kg i.p., 4-5 days	1.44	115 (14.4) ^e	1.07 (0.15) ^a	0.21 (0.03)	1.28 (0.71) ^a
	1.92	178 (19.0)	1.35 (0.18) ^a	0.36 (0.06)	1.71 (0.17) ^b
	2.80	578 (28.7) ^b	3.05 (0.62) ^a	0.96 (0.04) ^b	4.01 (0.89) ^b
BNF	0.43	112 (16.4) ^a	0.37 (0.15)	0.13 (0.09)	0.50 (0.23)
200 mg/kg i.p., 40 hours	0.86	369 (14.3) ^d	0.90 (0.15) ^a	0.40 (0.10) ^a	1.30 (0.41) ^a
	1.44	677 (10.1) ^{d,e}	1.41 (0.12) ^a	0.63 (0.09) ^c	2.11 (0.53) ^d

Results are given as mean (\pm s.e.m.), n = 6, except * n = 20.

Significantly different from untreated, same dose

a: P<0.05 b: P<0.01 c: P<0.005 d: P<0.001 e: significantly different from PB treated P<0.001.

1 Thioether vs MA excretion, $r_s = 0.74$, P<0.001.

2 Thioether vs PGA excretion, $r_s = 0.73$, P<0.001.

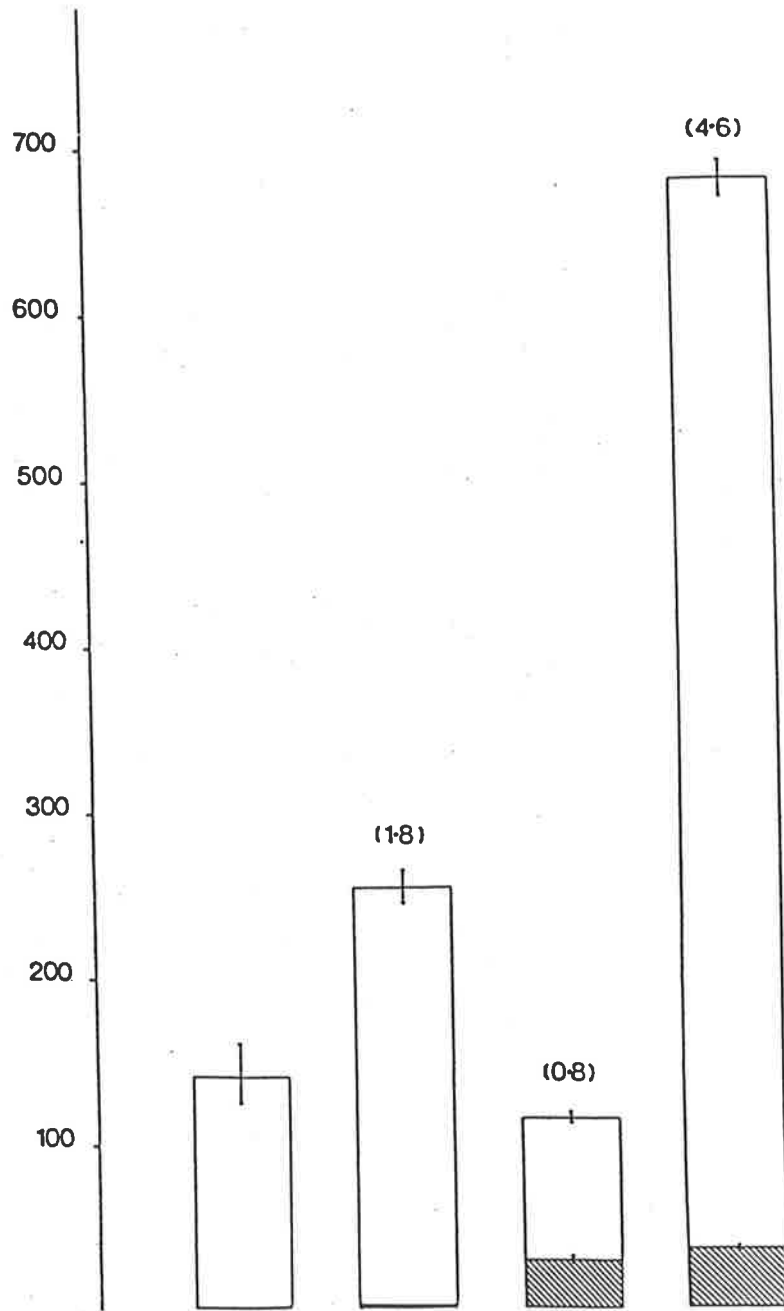
3 Thioether vs (MA+PGA) excretion, $r_s = 0.77$, P<0.001.

DIAGRAM 4.5

The effect of microsomal enzyme induction on basal (▨) and styrene-related (□) urinary thioether excretion of rats. Values are given as mean \pm s.e.m. (n = 6). Values in parentheses represent the magnitude of increased thioether excretion after accounting for inductive pretreatment effects on basal thioether excretion.

THIOETHER

$\mu\text{mol-SH}/\text{mmol creatinine}$
STYRENE 1.44 mmol/kg



STYRENE 0.43 mmol/kg

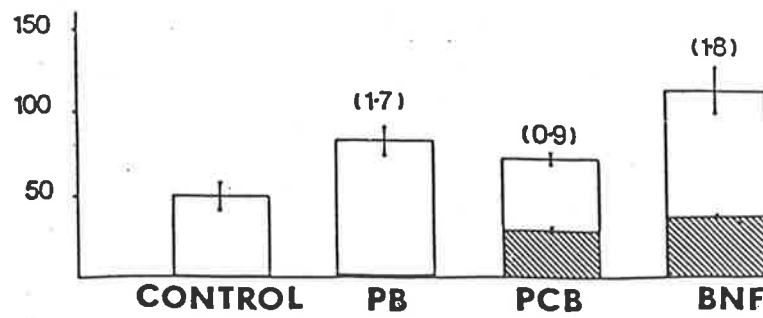


TABLE 4.6

Urinary thioether, mandelic acid (MA) and phenylglyoxylic acid (PGA) elimination in untreated rats 24 hours following administration of styrene. II. Chronic Styrene Treatment.

Dose (mmol/kg)	Week	Thioether ($\mu\text{mol-SH}/\text{mmol creatinine}$)	MA ¹	PGA ² (mmol/mmol creatinine)	(MA+PGA) ³
1.44	0	166 (8.13)	0.52 (0.12)	0.14 (0.04)	0.66 (0.14)
	1	180 (5.02)	0.48 (0.07)	0.15 (0.02)	0.63 (0.09)
	2	184 (9.44)	0.49 (0.03)	0.16 (0.01)	0.64 (0.04)
	3	144 (7.88) ^a	0.54 (0.02)	0.14 (0.003)	0.68 (0.02)
2.40	0	219 (8.53)	1.09 (0.02)	0.48 (0.06)	1.57 (0.07)
	1	219 (8.63)	0.84 (0.05)	0.30 (0.01)	1.13 (0.05)
	2	227 (17.9)	1.05 (0.03)	0.49 (0.01)	1.53 (0.03)
	3	191 (19.8)	0.89 (0.02)	0.44 (0.01)	1.33 (0.03)

Results are given as mean (\pm s.e.m.), n = 6.

a Significantly different from week 1, P<0.05.

1 Thioether vs MA excretion, $r_s = 0.82$, P<0.02.

2 Thioether vs PGA excretion, $r_s = 0.80$, P<0.02.

3 Thioether vs (MA+PGA) excretion, $r_s = 0.82$, P<0.02.

From the data presented in Table 4.5, BNF appeared to selectively enhance urinary thioether output, whereas other pretreatment regimes produced comparable magnitudes of increase in urinary thioether, MA and PGA excretion.

(2) Chronic studies

Styrene-related thioether output appeared consistent during chronic treatment of rats with either 1.44 mmol/kg or 2.40 mmol/kg of styrene. (Refer to Table 4.6.) Although administration of 1.44 mmol/kg styrene over a continuous three-week period resulted in a significant reduction in styrene-related thioether output (Wilcoxon matched-pairs signed-rank test, two-tail, $P < 0.05$), the magnitude of the decrease was small relative to concentrations of urinary thioethers excreted. Urinary MA and PGA elimination were not influenced by either treatment regime.

Discussion

Urinary thioether output of fibreglass workers was markedly enhanced following a working day exposure, a maximum increase of 571 $\mu\text{mol-SH}/\text{mmol creatinine}$ being reported. Although employees are exposed to a range of chemicals in the workplace, including solvents and cleaning agents, work-related increases in thioether elimination were probably largely attributable to styrene exposure, based on significant positive relationships demonstrated between urinary excretion of thioethers and specific styrene metabolites (MA, PGA).

On the basis of data obtained from animal studies, increases in styrene-related thioether excretion were shown to be dose-related.

However, quantitative interpretations of exposure-related effects on thioether output in occupational studies were somewhat limited due to the lack of data detailing ambient styrene concentrations. Qualitatively, thioether elimination of fibreglass workers tended to be exposure-related, as laminators, potentially exposed to very high styrene concentrations due to employment duties, excreted very high levels of thioethers. Thioether output of these workers was markedly greater than that of either petrol station attendants or research laboratory staff reported in Chapter 3, and other occupational groups monitored in previous studies (van Doorn et al. 1981a; van Doorn et al. 1981c; Jagun et al. 1982).

Elevations in work-related thioether output may be directly attributable to increased chemical exposure, as has been discussed, or may result from inductive effects of chemical exposure on microsomal monooxygenase activity. Previous studies by Vainio et al. (1979) and Savolainen & Vainio (1977) had suggested that chronic exposure to styrene can result in auto-induction of its own clearance via oxidative/mercapturate pathways. As all workers had been employed at least one year - most laminators at least five years - in their respective companies, this proposal may provide some explanation for work-related increases in thioether excretion. However, evidence from animal studies was not strongly in favour of this proposal.

A study of new rubber workers by Kilpikari & Savolainen (1982) reported initial decreases in work-associated thioether output after one month's employment, with significant elevations only observed after a continuous six month employment period. This effect could be explained by an initial inhibition of clearance of chemicals and/or metabolites via GSH conjugation, followed by reversal due to

compensatory increases in GSH-transferase activity. Results from chronic animal studies may therefore possibly be explained by inadequate duration of styrene treatment regimes. Alternatively, styrene may in fact stimulate its own biotransformation, but pathways induced may not necessarily involve clearance via GSH conjugation.

Work-related thioether elimination was enhanced amongst smokers. This result, consistent with smoking-related increases in thioether output demonstrated by studies of petrol station attendants and research laboratory staff, had been previously explained jointly by clearance of cigarette smoke components via GSH conjugation, and by inductive effects of cigarette smoke and constituents on microsomal monooxygenase activity as demonstrated by Van Cantfort & Gielen (1981).

Both general (PB, PCB) and cytochrome P-448-specific (BNF) inducing pretreatments increased styrene-related thioether, MA and PGA excretion by rats. However, whereas general enzyme inducers produced comparable magnitudes of increase in each parameter, pretreatment with BNF was shown to significantly enhance urinary thioether output relative to either MA or PGA excretion. This result tends to imply that both pathways catalyzed by microsomal monooxygenases - styrene 7,8-oxide and styrene 3,4-oxide formation, the latter postulated on the basis of studies by Pfäffli et al. (1982) - are sensitive to enzyme induction. Increased formation of styrene 3,4-oxide relative to 7,8-epoxide may significantly increase substrate availability for GSH conjugation and therefore explain selective enhancement of urinary thioether output observed. Therefore, microsomal enzyme induction, a phenomenon associated with cigarette smoking, is a feasible mechanism for the apparent increase in

work-related thioether output of smokers. Furthermore, selective induction of formation of styrene 3,4-epoxide, previously shown to be a potent mutagen (Watabe et al. 1982), may influence toxicity and hence potential health risks associated with occupational styrene exposure.

Monitoring of urinary thioether output in follow-up studies indicated that the technique could be routinely applied, in conjunction with MA and PGA quantitation, to provide daily estimates of worker exposure. Work-related thioether excretion of Company D employees was increased with progression of the week, corresponding to increased fibreglass spraying, moulding and boat construction resulting from company production routines. As consistently low thioether concentrations were excreted in pre-work samples, cumulative effects of styrene exposure on thioether output could be disregarded. The change in thioether index of Company B workers between studies illustrates how a change in factory hygiene can be readily monitored by this technique.

Interpretation of thioether data is, however, limited by characteristics of the assay system. Although increases in thioether output of workers were attributed to bioactivation of styrene, specific conjugates derived from either styrene 7,8- or styrene 3,4-epoxides could not be distinguished. Furthermore, relative extraction and hydrolysis efficiencies of conjugates generated are undefined at this stage.

C H A P T E R F I V E

GENERAL DISCUSSION

CHAPTER 5

General Discussion

Reproducibility of the thioether technique and its value as a biological monitor of exposure has been previously questioned by Pentz (1978) and Buffoni et al. (1982). Pentz had reported higher urinary thioether output by subjects not exposed to any appreciable concentrations of chemicals, with large intra-individual variations of at least 100%. Animal studies had indicated significantly higher thioether excretion amongst untreated rats than values reported in this thesis (refer to Chapter 2) and by van Doorn et al. (1980). Additionally, no evidence of significant differences between levels of thioethers excreted by untreated rats and those exposed to toxic chemicals such as N-nitrosamines, dimethylsulphate and benzo(α)pyrene was demonstrated.

Discrepancies arising between studies by Pentz (1978, 1979) and those of van Doorn et al. (1979, 1980, 1981a, 1981c) and this thesis can be explained by consideration of assay procedures adopted by each research group. In both animal and human studies Pentz had directly applied the Ellman assay to urine samples without attempting to correct for the presence of endogenous disulphides, such as cystine, or suppress auto-oxidation of thiols. However, the methodology of van Doorn et al. (1980), adopted in this thesis, incorporated an extraction procedure selective for mercapturates and other thioethers. High 'control' values reported by Pentz for both rats and man can therefore be attributable to the presence of significant concentrations of endogenous disulphides in urine samples.

Sedlak & Lindsay (1968) had previously studied optimal conditions

required for colour development of the Ellman reaction, and had shown that final pH of the reaction mixture must be within the range of 8.0 to 9.0, as intensity of colour produced outside this range was shown to be related to pH rather than sulphhydryl concentration. In experiments performed in this thesis pH of the assay system was monitored and maintained within 8.0 to 9.0. However, Pentz has given no account of such undertakings, large intra-individual variations therefore possibly being attributable to fluctuations in reaction pH.

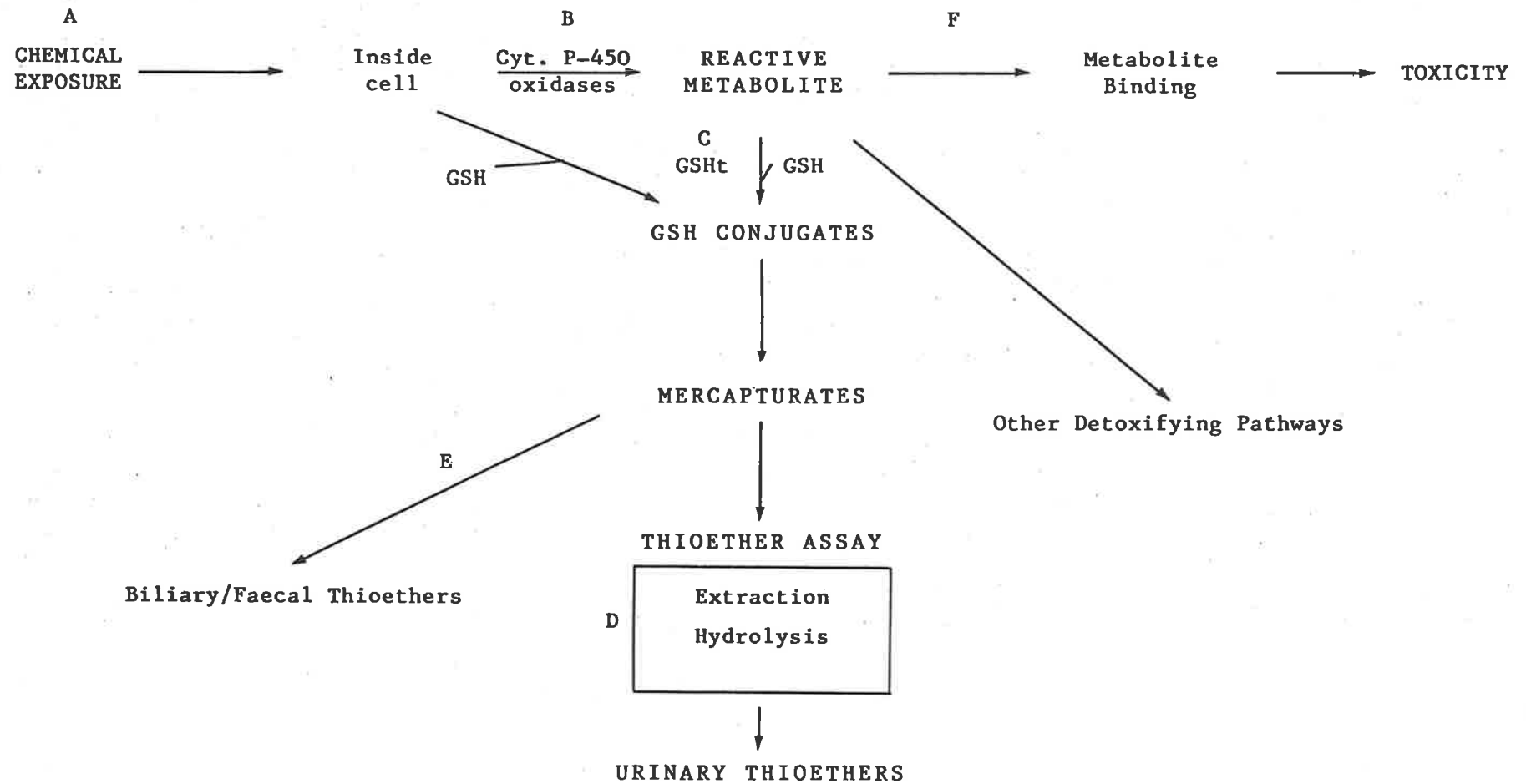
On the basis of experimental studies described in this thesis, quantitation of urinary thioether output was shown to fulfil necessary criteria of sensitivity and reproducibility* and was a simple, rapid means of monitoring chemical dosage or exposure. Major advantages of the technique are, firstly, simplicity of assay procedure and, secondly, convenience of non-invasive sample collection, enabling application as a means of assessing both chemical exposure in the workplace and efficiency of occupational hygiene practices. Determination of urinary thioether output, an example of a biological exposure test, may be a more reliable indicator of chemical bioavailability and could therefore be a useful inclusion within industrial health surveillance programmes.

Urinary thioether excretion has been shown to be dependent on both (A), magnitude of chemical exposure, and (B, C), relative activities of toxifying and detoxifying pathways of chemical biotransformation. (Refer to Diagram 5.1.) Animal studies have indicated dose-related influences on urinary thioether output. Evaluation of human data for possible exposure-response relationships was, however, more complex, the workplace generally offering multi-chemical exposure varying in intensity and duration.

*(Refer to Appendix 7)

DIAGRAM 5.1

A summary of major factors influencing urinary thioether output.



Previous studies by Vainio et al. (1978) and Kilpikari (1981) of rubber and chemical workers had suggested that work-related thioether output was dependent on the intensity of chemical exposure. In both studies the highest levels of thioethers were measured in urine samples of workers involved in rubber synthesis and manufacture and therefore potentially exposed to the highest concentrations of rubber additives, resorcinol and hexamethylene tetramine, via dermal or inhalation routes. Work presented in this thesis further substantiated dose- or exposure-response relationships for urinary thioether excretion by man. Driveway attendants, receiving greater exposure to petroleum fumes than employees of self-service outlets, excreted significantly higher levels of thioethers. Additionally, fibreglass laminators, potentially exposed to high concentrations of styrene vapours, excreted very high levels of thioethers, greater than concentrations eliminated by any other occupational group studied to date.

Urinary thioether output has been shown to be sensitive to induction of microsomal monooxygenase activity, the proposed 'rate-limiting step', (refer to Chapter 2), in chemical biotransformation. Changes in GSH-transferase activity also produced by inductive pretreatments were of relatively minor influence. Enhancement of microsomal enzyme activity, and hence reactive metabolite formation, may significantly deplete hepatic GSH stores and limit the ability of detoxification mechanisms. Previous studies by Mitchell et al. (1973) have shown that GSH conjugation is substrate-limited, irreversible covalent binding of reactive metabolites to cell nucleophiles occurring on depletion of endogenous GSH to less than 30% of normal levels (as produced by toxic doses of paracetamol). Therefore, marked

elevation in work-related thioether output - for example, to 574 $\mu\text{mol-SH}/\text{mmol creatinine}$ by one fibreglass laminator - suggests the possibility of incomplete chemical detoxification and initiation of metabolite-mediated toxicity.

Concentrations of thioethers measured in samples are dependent on efficiencies of extraction and hydrolysis of chemicals (D, Diagram 5.1). Although assumed that the efficiency of each step may be comparable for most compounds, recoveries of different mercapturates may vary markedly. Preliminary studies in our laboratory (White, personal communication) have confirmed significant variability in the recovery of mercapturate and non-mercapturate thioethers. However, generally the influence of methodological variables on interpretations of thioether data is minor relative to the influence of biological factors (van Doorn et al. 1981b).

Animal studies have demonstrated that the rat was an appropriate model for evaluating sensitivity and reproducibility of the thioether technique and investigation of parameters modifying thioether excretion in man. However, extrapolation from this animal model to a complex human exposure situation was limited by quantitative differences in (a) activity of toxifying and detoxifying pathways (B, C), and (b) the balance between urinary and biliary/faecal excretion (E); this being particularly relevant for chemicals of high molecular weight. Consequently, Summer et al. (1979) and Rozman et al. (1982) have proposed that primates, such as chimpanzees or rhesus monkeys, possessing comparable activities of GSH-transferases as man (Hayakawa et al. 1974), may provide a more suitable animal model for quantitatively evaluating factors influencing thioether output by man.

The major limitation of the thioether technique, previously

discussed in Chapters 2 and 3, is that it is nonselective and cannot identify specific chemicals as sources of thioether substrates nor differentiate mercapturates arising from direct conjugation with GSH or indirectly via electrophilic oxidative metabolite formation. Inferences regarding the sources of work-related increases in thioether output can be made by (a) isolation of workers from situations involving significant chemical exposure, (b) simultaneous monitoring of specific chemical metabolites in body fluids, and (c) monitoring of ambient pollutant concentrations within the workplace. Obviously improved specificity of the assay system, initiated by Fahey et al. (1981) and Hernandez et al. (1982), would aid interpretation of thioether excretion data. However, further developmental work and validation of each assay system is required before either approach may be routinely applied within industry.

Ariëns (1980) has proposed that nonspecificity of the thioether assay is instead advantageous, as increases in work-related thioether output are indicative of increased clearance of detoxification products of cytotoxic or genotoxic events. As discussed in Chapter 1, reactive electrophilic metabolites generated by microsomal oxidation are capable of alkylating cell nucleophiles, the primary step in initiation of cell lesions. Determination of urinary thioether excretion may be a sensitive index or 'signal' of excessive chemical exposure within the workplace. Marked enhancement of thioether output following the working day may suggest significant formation of alkylating metabolites and possibly implicate initiation of metabolite-mediated genotoxic lesions. (Refer to Diagram 5.1, F.)

Absence of increased thioether elimination following the working day does not automatically imply that either nil or negligible chemical

exposure has occurred. Such a result may be a consequence of one or more of the following factors: (a) inappropriate sampling strategies, particularly relevant for chemicals with short biological half-lives, (b) alternative pathways for chemical metabolism not involving GSH conjugation, (c) poor recoveries of mercapturates from extraction and hydrolysis stages of the thioether assay, particularly relevant for small aliphatic compounds (White, personal communication).

The thioether technique is, however, not complete, as assessments of health hazards associated with occupational chemical exposure based on thioether excretion data are somewhat tenuous. Instead, thioether measurements may be used for routine screening of occupational groups, significant elevations in thioether output signalling the need for further specialized cytogenetic evaluation and regulation of ambient pollutant concentrations in the working environment. In summary, the thioether technique has been shown to be a simple and useful index of occupational chemical exposure and could be readily incorporated into comprehensive industrial surveillance programmes to 'signal' areas of potential health hazard to workers.

A P P E N D I C E S

APPENDIX 1

Determination of Urinary Thioether Concentration

1. Standard Curve N-Acetylcysteine (N.A.C.)

No extraction.

No hydrolysis.

Concentration N.A.C. (μM)	Absorbance 412 nm	Corrected Absorbance 412 nm
0	0.07	0.03
25	0.10	0.06
50	0.125	0.085
100	0.195	0.155
250	0.375	0.335
500	0.665	0.625

Absorbance 412 nm urine blank = 0.03.

Absorbance 412 nm DTNB blank = 0.01.

2. Standard Curve N.A.C. - After Extraction

Concentration N.A.C. (μM)	Absorbance 412 nm	Corrected Absorbance 412 nm
0	0.07	0.03
25	0.085	0.045
50	0.115	0.075
100	0.15	0.11
250	0.295	0.255
500	0.525	0.485

Efficiency of extraction:

$$500 \mu\text{M N.A.C. after extraction} \frac{0.485}{0.625} \times 100 = 78\%$$

3. Standard Curve N.A.C. - After Hydrolysis

Concentration N.A.C. Added (μM)	Absorbance 412 nm	Corrected Absorbance 412 nm
0	0.07	0.03
25	0.075	0.035
50	0.09	0.05
100	0.09	0.05
250	0.155	0.115
500	0.225	0.185

Absorbance 500 μM added = 0.185.

Dilution factor = 2.

$$\therefore \text{Efficiency of hydrolysis} = \frac{2 \times 0.185}{0.485} \times 100 = 76\%$$

Calculation of Thioether Concentration

Thioether

$$= 2 \left[\begin{array}{l} \text{Posthydrolysis x hydrolysis} \\ \text{concentration efficiency} \\ \text{(B)} \end{array} \right] - \left[\begin{array}{l} \text{Prehydrolysis x extraction} \\ \text{concentration efficiency} \\ \text{(A)} \end{array} \right]$$

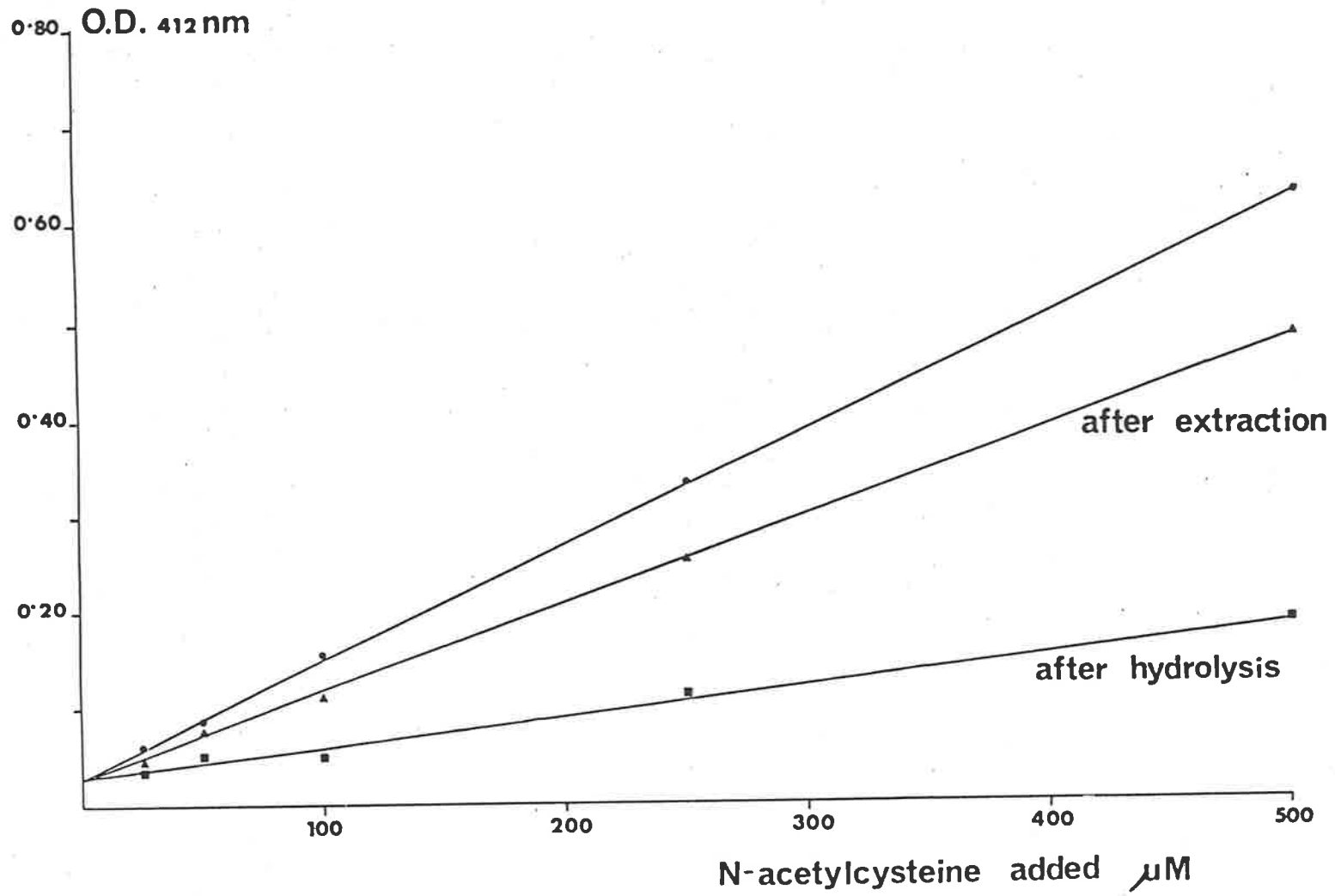
$$= 2 [B \times 1.32] - [A \times 1.28] \mu\text{M}.$$

Thioether ($\mu\text{mol-SH}/\text{mmol creatinine}$)

$$= \frac{\text{Thioether } \mu\text{M}}{\text{Creatinine mM}}$$

APPENDIX 1

Standard curves for N-acetylcysteine (μM), before and after extraction and hydrolysis, respectively.



APPENDIX 2

Determination of Urinary Creatinine Concentration

(Reference: Yatzidis H (1974), Clin. Chem. 20(9), 1131-1134.)

Reagents

1. Saturated picric acid 15 g/L distilled water.

2. Phosphate buffers.

pH 10.0 1 M NaH_2PO_4 490 ml

1 M NaOH 510 ml

pH 11.5 1 M NaH_2PO_4 385 ml

1 M NaOH 615 ml

3. Alkaline picrate reagents:

A: 1 volume picric acid

4 volumes phosphate buffer, pH 10.0.

Mix immediately prior to use, final pH = 9.65.

B: 1 volume picric acid.

4 volumes phosphate buffer, pH 11.5.

Mix immediately prior to use, final pH = 11.5.

Procedure

1. Dilute urine 20 times with distilled water.

2. To a duplicate series of tubes add

(1) 3.0 ml reagent A + 0.4 ml diluted sample,

(2) 3.0 ml reagent B + 0.4 ml diluted sample.

3. Incubate at 37°C for 45 minutes.

4. Read absorbance 500 nm.

5. Creatinine concentration is linearly related to the absorbance difference (B-A).

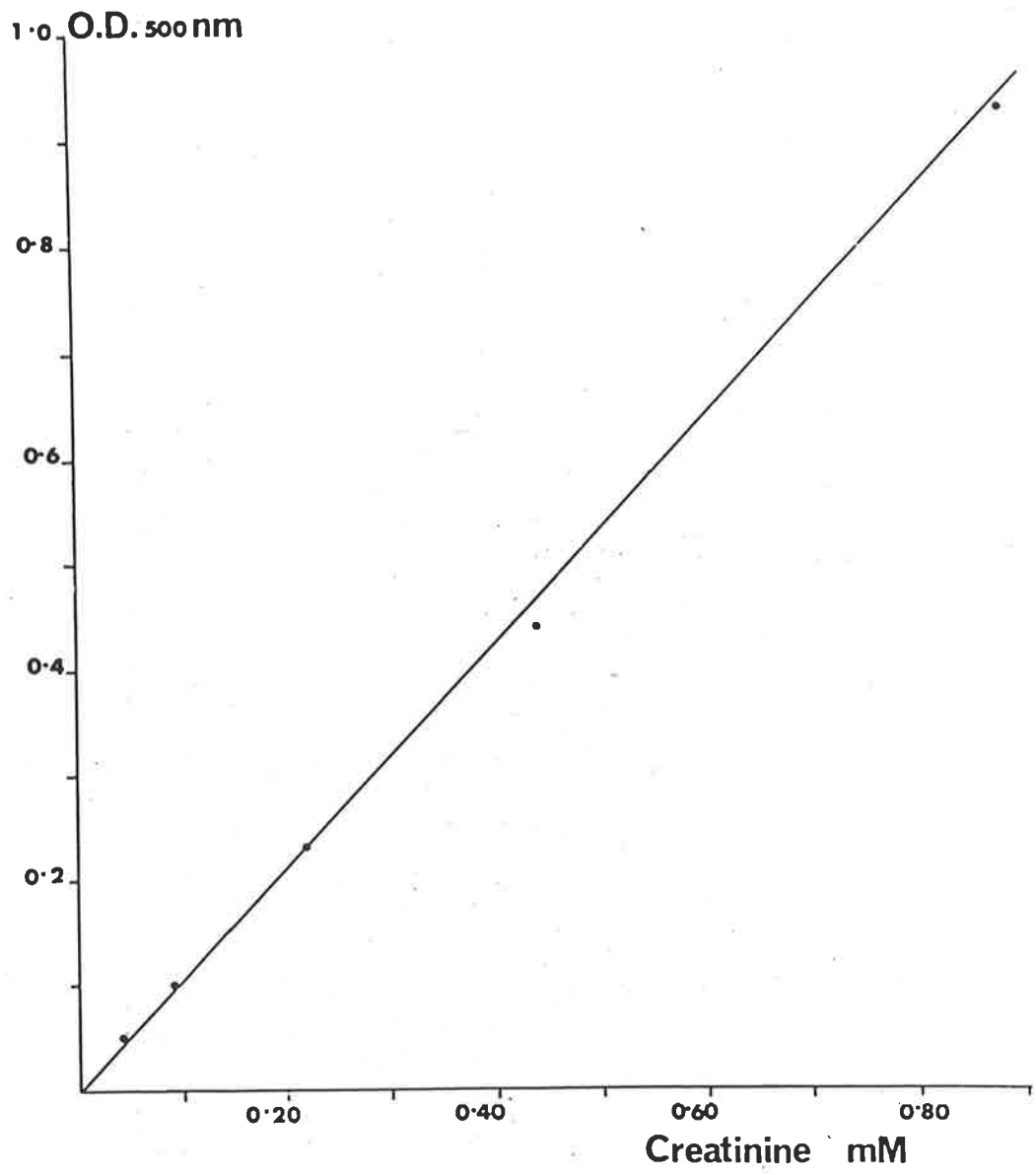
Standard Curve

Creatinine Concentration	mg/100 ml	mM
	0.50	0.04
	1.00	0.09
	2.50	0.22
	5.00	0.44
	7.50	0.66
	10.0	0.88

1. A duplicate series of tubes were prepared and reagents A and B added to each series.
2. Tubes were incubated at 37°C for 45 minutes.
3. Absorbance at 500 nm was read.
4. Creatinine concentration was linearly related to the absorbance difference (B-A).

APPENDIX 2

Standard curve relating optical density (O.D. 500 nm) to the difference in creatinine concentration (B-A, mM).



APPENDIX 3

Determination of GSH-S-Transferase Activity

(Reference: Habig WH, Pabst MJ, Jacoby WB (1974),

J. Biol. Chem. 249, 7130-7139)

Incubation Mixture

Phosphate buffer (100 mM, pH 6.5)	2.50 ml
Glutathione, reduced (10 mM, 3.07 mg/ml water)	0.40 ml
1, chloro-2,4 dinitrobenzene (CDNB) (4.8 mM, 8.1 mg/ml ethanol)	0.10 ml
S-9 fraction (500 µg/ml)	<u>1.00 ml</u>
	<u>4.00</u>

Procedure

1. Pre-incubate each tube for 2 minutes at 37°C.
2. Start the reaction by adding 0.1 ml CDNB, mix.
3. Incubate for 2 minutes at 37°C.
4. Immediately transfer contents of incubation tube to a quartz cuvette and read absorbance at 340 nm.

Note: Each sample is read against a reference cell which has the complete incubation mix except for CDNB.

Standard Curve

nmoles conjugate*	0	1	5	10	50	100	250	500
µl conjugate stock*	0	1	5	10	50	100	250	500
ml phosphate buffer	2.6	2.599	2.595	2.59	2.55	2.5	2.35	2.1

1. Add reduced GSH 0.4 ml

S-9 Fraction 1.0 ml

2. Read absorbance at 340 nm against a reference cell containing all constituents of the incubation mix except CDNB.

* CDNB conjugate (S-glutathione-CDNB) 4.75 mg/10 ml water.

APPENDIX 3

Standard curve relating optical density (O.D. 340 nm) to concentration of S-glutathione-DNB (nmoles/incubate).

1.5 O.D. 340nm

1.0

0.5

100

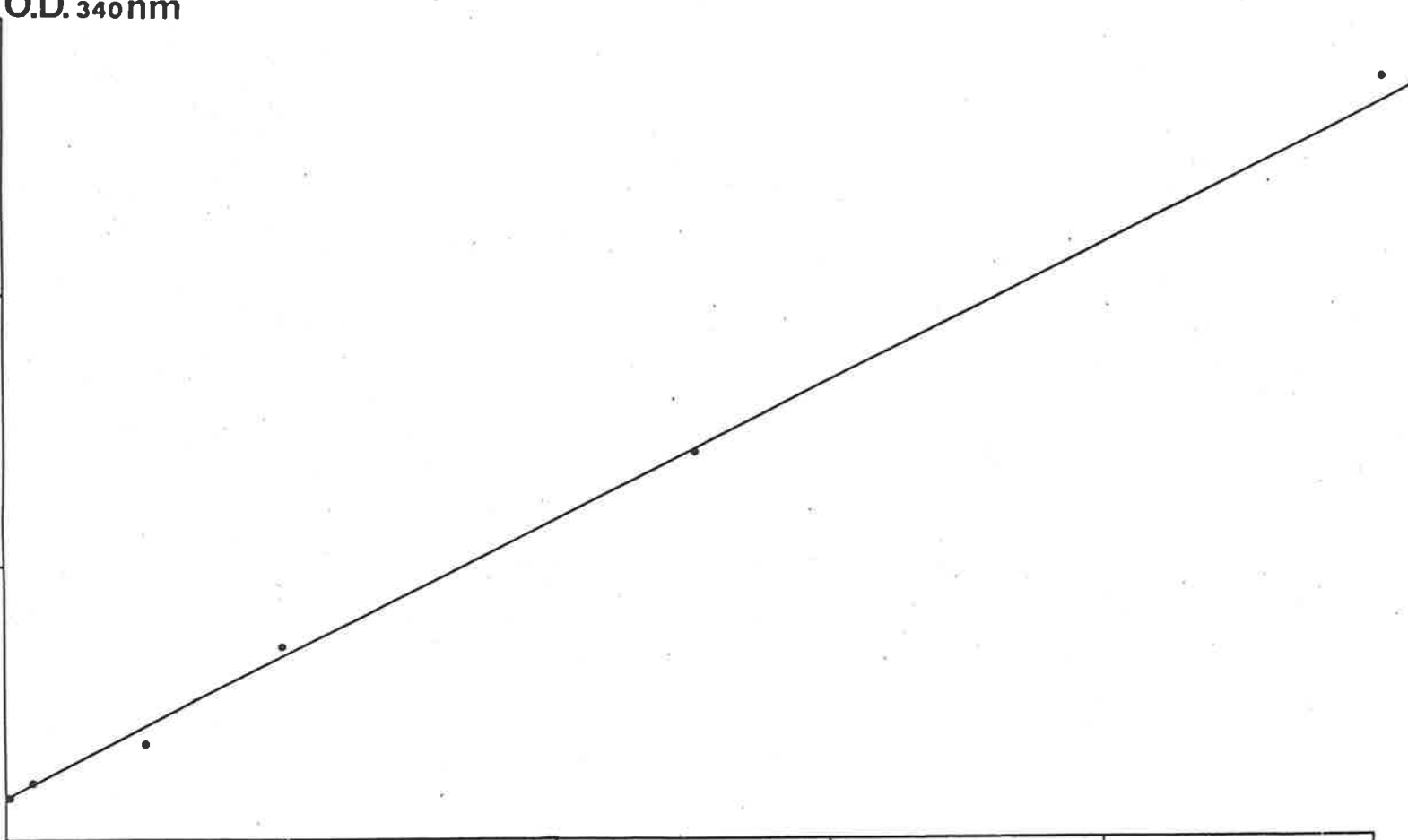
200

300

400

500

nmol/incubate S-Glutathione DNB



APPENDIX 4

OCCUPATIONAL CHEMICAL STUDY

Thank you for agreeing to participate in this study. In addition to providing samples of urine as specified below, we ask you to provide us with some personal data, which will allow us to categorise the data and enable us to determine whether dietary factors or social habits may influence the results.

ALL INFORMATION GIVEN WILL BE TREATED AS CONFIDENTIAL, AND YOU WILL NOT BE IDENTIFIED BY NAME IN ANY REPORT OF THE RESULTS.

SAMPLE COLLECTION

You may collect the urine samples in any clean container, or directly into the bottle provided. It is not necessary to fill the container. A sample as small as 30 ml (approx. 1/2 cup) will do.

The morning sample should be the first voiding after getting up in the morning. (Remember to take the sample bottle home with you.)

The evening sample should be taken preferably around 7.00 p.m., but the time is not critical, and it may be taken any time from 0-4 hours after finishing work.

Please make sure that you write on the label of each bottle the following information:

1. Name (or initials).
2. Date and time of sample collection.

PERSONAL DETAILS

Name

SEX AGE WT

DATE(S) & TIME(S) AT
WHICH SAMPLES WERE
TAKEN

PLACE OF WORK

NATURE OF WORK

DO NOT WRITE IN THIS AREA

Identification No.

Sample Code

Creatinine

Thiol

Thioether

Do you know what types of chemicals (e.g., solvents, fumes, vapours, dusts, pesticides, etc.) you may have been exposed to

- (a) at work
- (b) at home

DIET/SOCIAL/MEDICAL

Do you smoke cigarettes / cigars / pipe ? If so, approximately how many per day (circle one):

None 1-10 10-20 More than 20

Please indicate approximate amounts of the following beverages that you normally consume per day:

Coffee	Carbonated soft-drinks (e.g., coke)	Beer
Tea	Cordials	Wine
Milk	Fruit juices	Spirits
Other		

Are you currently taking any medicines? If so, please specify:

.....

If any queries about sampling procedures arise

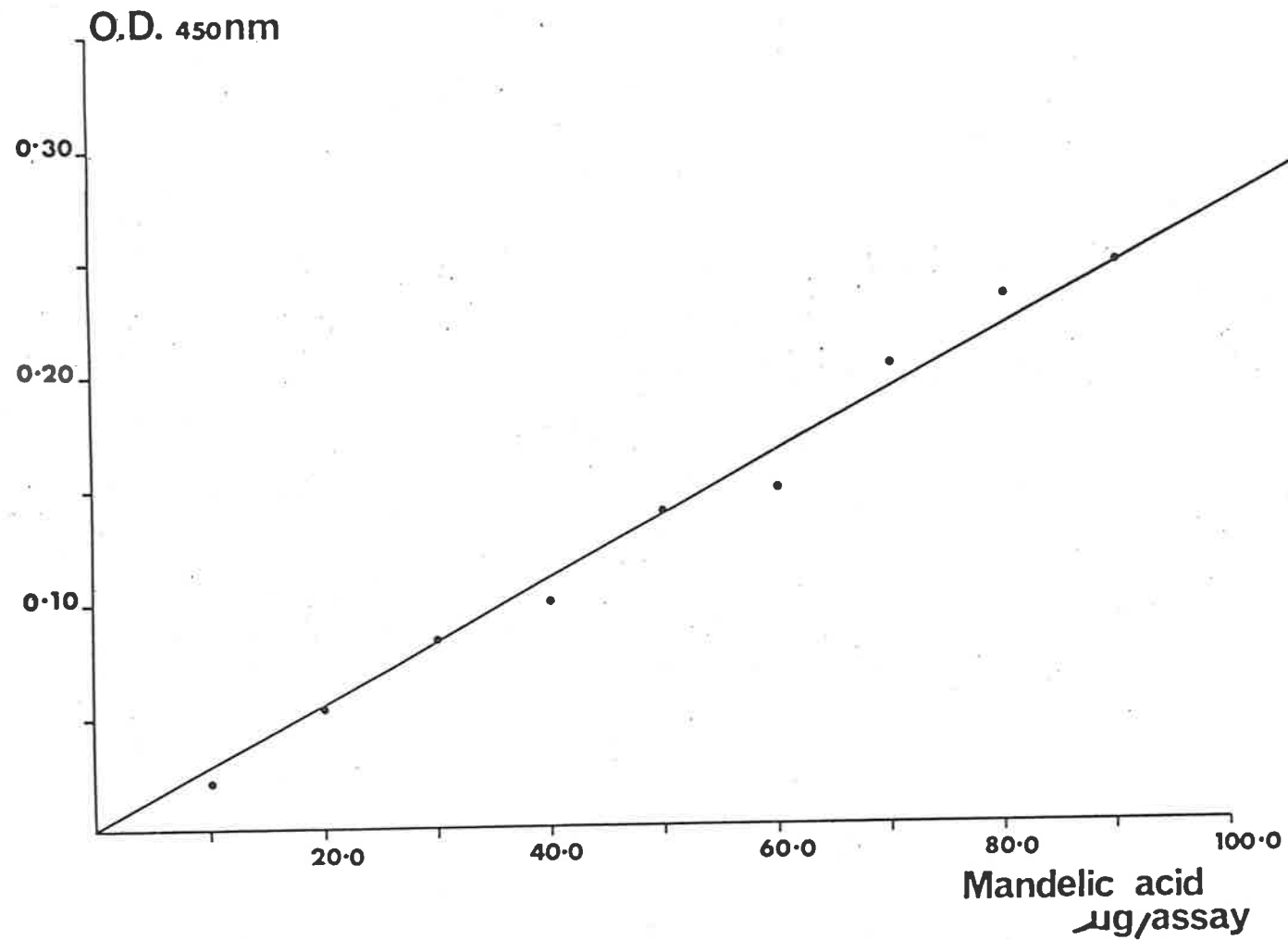
Telephone Dr Priestly: 228-5287 (office)
79-7525 (a.h.)

or

Miss J. Stock: 228-5985 (office)

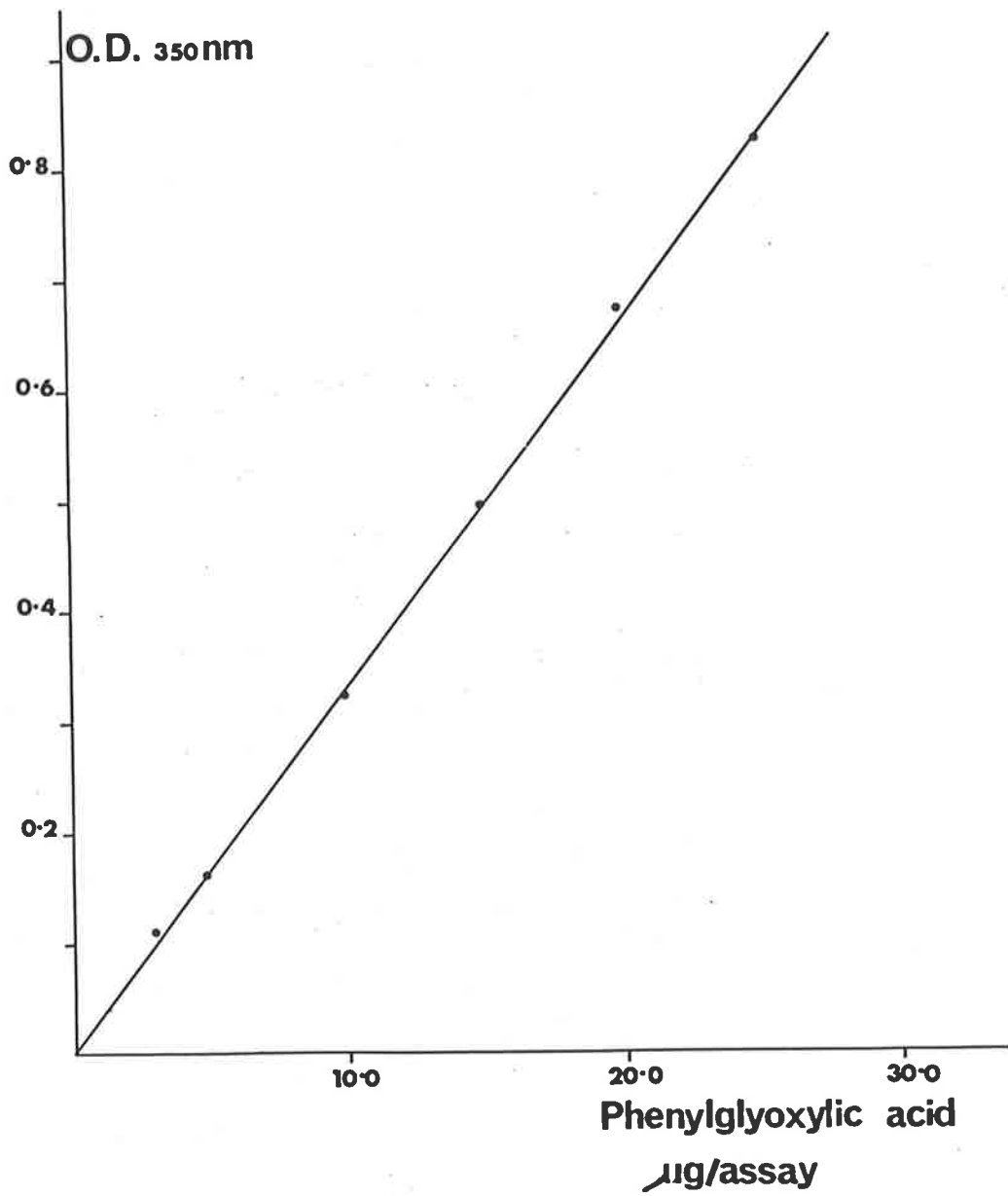
APPENDIX 5

Standard curve for mandelic acid (MA). The optical density at 450 nm is plotted against the amount of MA (μg)/assay tube.



APPENDIX 6

Standard curve for phenylglyoxylic acid (PGA): The optical density at 350 nm has been plotted against the amount of PGA (μg)/assay tube.



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Validation of Assay Reproducibility :

- Thioether
- Creatinine
- Phenylglyoxylic acid
- Mandelic acid

A

Evaluation of assay reproducibility and reliability was made by determination of the coefficients of variation of thioether, creatinine, phenylglyoxylic acid and mandelic acid assays, derived from 6 or 7 standard curves constructed on different experimental days.

Standard curves were constructed as described in Chapters 2, 4 and Appendices 1, 2. Absorbance readings for thioether determination were made within 1 hour of addition of Ellman's reagent. Determination of styrene metabolite levels was made 30 minutes following addition of sulphuric acid/formalin reagent.

Values for each assay coefficient of variation are given as a mean of coefficients of variation of at least 5 points of the standard curve repeated 6 or 7 times \pm standard error of mean.

1.Thioether Variability

- a)N-acetylcysteine added, no extraction, no hydrolysis =3.22 \pm 1.13%
- b)Extraction step included =2.18 \pm 0.91%
- c)Complete analytical procedure, extraction plus hydrolysis
4.95 \pm 1.51%

Standard curve repeated 7 times for each estimate.

The overall mean assay coefficient of variation =3.45 \pm 0.80%

2.Creatinine Variability

Coefficient of variation of assay =2.77 \pm 0.70%

3.Phenylglyoxylic acid Variability

Coefficient of variation of assay =5.21 \pm 1.81%

4.Mandelic acid Variability

Coefficient of variation of assay =8.61 \pm 1.91%

Standard curve repeated 6 times for creatinine, phenylglyoxylic acid and mandelic acid estimates.

B

Additional evidence of assay reproducibility and reliability was provided with reference to both animal and human data.

1. Urinary thioether output of control rats was measured over 4 consecutive days to investigate possible sources of variation of thioether output and measurement. The following values were obtained:

Day 1 : 0.44 ± 0.03 $\mu\text{mol-SH}/\text{mmol creatinine}$

Day 2 : 0.445 ± 0.03 "

Day 3 : 0.43 ± 0.02 "

Day 4 : 0.43 ± 0.02 "

(Values given as mean \pm s.e.m., n=20)

These mean values lie within the reported range given in Chapter 2, Table 2.1, i.e. 0.44 ± 0.01 . The consistency of thioether output tended to indicate that either sampling period or technical procedures employed do not significantly influence thioether measurement.

2. Urine samples obtained from styrene workers with high levels of thioethers determined on initial analysis, were reanalyzed following a three month interval. Duplicate values obtained are shown in Table 7.1.

Although the data is insufficient to enable statistical analysis (n=2), duplicate values obtained upon reanalysis are consistent with thioether phenylglyoxylic acid and mandelic acid values found upon initial analysis, and variation between duplicates on the same analysis day is of a similar order. Thioether and styrene metabolite levels tend to remain constant, therefore, after storage.

Evidence has therefore been presented both a), directly by assay coefficients of variation, and b), indirectly by reanalysis of control and work-exposed samples, that assays used are reproducible and reliable. Day-to-day variation in analytical procedures is not a significant factor influencing thioether and styrene metabolite levels measured.

TABLE 7.1

SUBJECT NO.	INITIAL ANALYSIS				REANALYSIS			
	Thioether		MA	PGA	Thioether		MA	PGA
	am	pm			am	pm		
5	1.43	11.8	0.82	0.10	1.44	11.7	0.80	0.10
	1.43	11.8	0.82	0.10	1.51	11.7	0.80	0.10
7	2.66	44.3	1.83	0.26	2.65	44.2	1.90	0.30
	2.66	44.3	1.83	0.26	2.65	44.2	1.90	0.30
8	1.08	48.3	2.30	1.78	1.05	50.0	2.30	1.75
	1.04	48.2	2.30	1.78	1.05	50.0	2.30	1.75
9	3.16	574	10.0	8.20	3.16	576	9.65	8.10
	3.16	574	10.0	8.20	3.16	576	9.70	8.10
12	1.04	13.9	1.16	0.55	1.05	14.0	1.14	0.51
	1.04	13.9	1.16	0.55	1.05	14.0	1.16	0.51
13	2.05	27.7	1.76	1.16	2.08	28.0	1.75	1.16
	2.05	26.9	1.76	1.16	2.08	28.0	1.75	1.16
14	1.91	24.5	0.58	0.25	1.89	24.5	0.60	0.25
	1.91	24.5	0.58	0.25	1.92	24.5	0.60	0.25
15	2.10	81.8	5.37	3.67	1.90	78.5	5.40	3.75
	2.00	81.8	5.37	3.67	1.90	76.7	5.40	3.75

Thioether given as $\mu\text{mol-SH}/\text{mmol creatinine}$

MA and PGA given as $\text{mmol}/\text{mmol creatinine}$