THE GLUTATHIONE S-TRANSFERASES: INHIBITION, ACTIVATION, BINDING AND KINETICS.

by

Alfred Ernst Adolf Thumser
B.Sc.(Med.)(Hons.)

A thesis submitted to the University of Cape Town Faculty of Medicine in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in the department of Medical Biochemistry.

August, 1990

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

Acknowledgements.

I would like to express my sincere appreciation to the following people:

My supervisors, Associate Professor Kathryn M. Ivanetich and Dr. Paul A. Adams, for their guidance, constructive criticism and motivation.

Dr. Richard Goold, a Ph.D. graduate from our laboratory at the time, for his help and assistance, especially with reference to computer analysis of data.

The head, Professor G.G. Harrison, and staff of the Department of Anaesthetics, Grootte Schuur Hospital, for their assistance with work related to halothane.

The staff of the Chemical Pathology laboratory, Red Cross War Memorial Children's Hospital for access to and assistance with the use of the Multistat Centrifugal Analyzer.

For assistance related to various aspects of the thesis I would also like to thank Dr. Melanie Ziman, Dr. Cynthia Sikakana, Susan Phillips, Jo-Anne Harper, Sadia Titus, Henry Terblanche and all the other helping hands.

I would like to gratefully acknowledge the financial assistance of the South African Medical Research Council and the University of Cape Town Postgraduate Bursary fund.

Abbreviations

A	Absorbance
Bis-acrylamide	N,N′-Methylenebisacrylamide
BSA	Bovine serum albumin
BSP	Bromosulfophthalein
CD	Circular dichroism
CDF	2-Chloro-1,1-difluoroethylene
CDNB	1-Chloro-2,4-dinitrobenzene
CM-cellulose	Carboxymethyl cellulose
CTF	2-Chloro-1,1,1-trifluoroethane
DBE	1,2-Dibromoethane (Ethylene dibromide)
DCNB	1,2-Dichloro-4-nitrobenzene
DEAE-cellulose	Diethylaminoethyl cellulose
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTE	Dithioerythreitol
DTNB	2,2′-Dinitro-5,5′-dithiobenzoic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
μM	Micromolar (10^{-6} M)
g	Standard acceleration of gravity
GSH	Glutathione
GSSG	Reduced glutathione
GST	Glutathione S-transferase
His	Histidine
HPLC	High performance liquid chromatography
HSA	Human serum albumin
K_D	Dissociation constant
K_i	Inhibition constant
K_m	Michaelis-Menten constant
M	Molar, $mol.dm^{-3}$
mM	Millimolar (10^{-3} M)
MP	Microperoxidase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PB	Phenobarbital
RNA	Ribonucleic acid
rpm	Revolutions per minute
S.D.	Standard deviation
SDS	n-Dodecylsulfate sodium salt
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
$t_{½}$	Half-life of a reaction
TCA	Trichloroacetic acid
TEAE-cellulose	Triethylamino ethyl cellulose
TEMED	N,N,N′,N′-tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)-aminomethane
UV	Ultraviolet
V_{max}	Maximum velocity of an enzyme reaction
ϵ	Extinction coefficient ($M^{-1}.cm^{-1}$)

List of Publications.

1. Ivanetich, K.M. & Thumser, A.E.A.; Inhibition of the GSH S-Transferase Isoenzymes by Ethylene Dibromide.; Liver Update **2**, 33-36, eds Kirsch, R.E., Kruskaly, J.B., Comos, G. & Terblanche, J., Balliere Trundall, London (1984).

2. Ivanetich, K.M., Thumser, A.E.A., Ziman, M.R., Baruch, Y. & Kirsch, R.E.; in "The Liver Annual **5**., Chapter 4: Liver Injury and Regeneration.", 71-115, eds Arias, I.M., Frenkel, M. & Wilson, J.H.P., Elsevier Science Publishers B.V. (1986).

3. Ivanetich, K.M., Thumser, A.E.A., Ziman, M.R., & Kirsch, R.E.; in "The Liver Annual **6**., Chapter 4: Liver Injury and Regeneration.", 91-162, eds Arias, I.M., Frenkel, M. & Wilson, J.H.P., Elsevier Science Publishers B.V. (1987).

4. Ivanetich, K.M. & Thumser, A.E.A.; Reversible Inhibition and Activation of Hepatic GSH S-Transferases by Ethylene Dibromide.; Pharmacol. Ther. **33**, 85-88 (1987).

5. Ivanetich, K.M., Thumser, A.E.A. & Harrison, G.G.; Halothane: Inhibition and Activation of Rat Hepatic Glutathione S-Transferases.; Biochem. Pharmacol. **37**, 1903-1908 (1988).

6. Adams, P.A., Byfield, M.P., Goold, R.D. & Thumser, A.E.; Kinetics of Heme Octapeptide (Microperoxidase-8 - MP-8) Formation Studied by High-Pressure Liquid Chromatography (HPLC) Monitoring of the Peptic and Tryptic Hydrolysis of Horse Heart Cytochrome-*c*.; J. Inorg. Biochem. **37**, 55-59 (1989).

7. Adams, P.A., Goold, R.D. & Thumser, A.A.; Heme-Peptide Protein Interactions - The Binding of Heme Octa and Undecapeptides, and Microperoxidase-8 and Microperoxidase-11, to Human Serum Albumin.; J. Inorg. Biochem. **37**, 91-103 (1989).

8. Adams, P.A., Goold, R.D. & Thumser, A.E.; Haem Peptide-Protein Interactions. Part 2. - Kinetics and Mechanism of the Interaction of Microperoxidase-8 with Apomyoglobin.; J. Chem. Soc. Faraday Transact. **85**, 3845-3852 (1989).

9. Ivanetich, K.M., Thumser, A.E.A., Phillips, S.E. & Sikakana, C.N.T.; Reversible Inhibition of Rat Hepatic GSH S-Transferase 1-2 by Bilirubin; Biochem. Pharmacol. (1990, in press).

List of Presentations.

1. Ivanetich, K.M. & Thumser, A.E.A.; Reversible Inhibition and Activation of GSH S-Transferases by 1,2-Dibromoethane; International Symposium on Clinical and Basic Aspects of Enzyme Induction and Inhibition, Essen, FRG (1985).

2. Thumser, A.E.A., Ivanetich, K.M. & Harrison, G.G.; Halothane: Multiple Effects on Hepatic Glutathione S-Transferases; The First Joint Congress of the South African Biochemical Society, South African Genetics Society and the South African Society for Microbiology (1986).

3. Ivanetich, K.M., Thumser, A.E. & Sikakana, C.N.T.; Reversible Inhibition of the GSH S-Transferases by Bilirubin; South African Biochemical Society, Ninth Congress (1988).

4. Adams, P.A., Goold, R.D. & Thumser, A.E.A.; The Physical and Inorganic Biochemistry of Microperoxidase-8 (MP-8). (7) The Interaction of MP-8 with Apo Myoglobin; South African Biochemical Society, Ninth Congress (1988).

5. Adams, P.A., Goold, R.D. & Thumser, A.E.A.; The Physical and Inorganic Biochemistry of Microperoxidase-8 (MP-8). (4) Interaction of MP-8 with Human Serum Albumin; South African Biochemical Society, Ninth Congress (1988).

Summary

The glutathione S-transferases are a group of xenobiotic detoxifying enzymes found in most plants and animals (Boyer & Kenney, 1985; Mannervik & Danielson, 1985). These proteins catalyze the conjugation of glutathione with a wide range of electrophilic compounds in the first step of their metabolism by the mercapturic acid pathway (Habig, 1983). In addition, these enzymes may act as hepatic transport proteins for endogenous compounds such as bilirubin. Aspects of the kinetics, inhibition, activation and ligand binding of the glutathione S-transferases are reported in this thesis; The focus was on the anesthetic halothane and an analogue, 1,2-dibromoethane, and subsequently on bilirubin and the heme peptides, microperoxidases 8, 9 and 11.

The volatile anesthetic agent halothane has been shown to decrease hepatic bromosulfophthalein uptake after multiple exposures (Biebuyck *et al*, 1970). As bromosulfophthalein uptake is thought to be a function of the glutathione S-transferases (Boyer & Kenney, 1985; Boyer, 1989), we decided to study the effect of halothane on these enzymes. *In vivo*, a single exposure of rats to halothane did not significantly decrease hepatic glutathione S-transferase levels. In contrast, multiple exposures to halothane initially decreased, and subsequently increased, glutathione S-transferase activity. This effect was isoenzyme-specific with enzymes containing subunits 3 and 4 being affected, although the possible effect on subunits 1 and 2 is debatable. *In vitro*, inhibition of cytosolic glutathione S-transferases was apparently irreversible and concentration- and time-dependent, with apparent half-maximal inhibition at 15 mM halothane and a half-life of *ca* 5 min. Inhibition was isoenzyme-dependent as isoenzymes 3-3, 3-4 and 4-4, but not isoenzymes 1-1 and 1-2, were affected. Halothane did not inhibit glutathione S-transferase activity in cytosol and the protective factor could not be removed by dialysis or hepatic perfusion. 1,2-Dibromoethane, but not halothane, was metabolized by the cytosolic glutathione S-transferases. *In vitro*, isoenzyme 1-2 was activated in a reversible process with a short half-life.

The halothane analogue, 1,2-dibromoethane, is known to inhibit glutathione S-transferase activity *in vivo* (Botti *et al*, 1982) and *in vitro* (Ivanetich *et al*, 1984). In conjunction with the investigation of the reversible effect of halothane *in vitro*, the effect of 1,2-dibromoethane was also investigated. 1,2-Dibromoethane reversibly activated and inhibited rat hepatic glutathione S-transferases. Isoenzymes 3-3 and 4-4 were activated in an uncompetitive manner with respect to

GSH. However, under conditions of CDNB variation these isoenzymes were inhibited at low, and activated at high, CDNB concentrations. These phenomena are explained in simplistic terms, but a comprehensive mechanistic scheme could not be formulated.

The glutathione S-transferases may act as hepatic transport proteins for non-substrate ligands such as bilirubin which are also reversible inhibitors of the glutathione S-transferases (Boyer, 1989). In several publications by Vander Jagt *et al* (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982, 1983) a system of "enzyme memory" has been proposed to explain the apparently complex inhibition kinetics of bilirubin and the protective effect of proteins. Our interpretation of the inhibition kinetics observed by Vander Jagt *et al* (Vander Jagt *et al*, 1982, 1983) differed from that of the authors. The process of bilirubin inhibition and protection by proteins was thus investigated. In our work, inhibition of isoenzyme 1-2 by bilirubin was determined to be a pseudo first-order process with a calculated second-order rate constant of approximately $5.2 \times 10^3\ M^{-1}.s^{-1}$. The addition of proteins to incubated mixtures of glutathione S-transferases and bilirubin prevented inhibition. Sephadex chromatography was used to demonstrate that the proteins bound high molar ratios of bilirubin and, in conjunction with inhibition experiments, the protective effect was shown to depend on protein identity and concentration. The hypothesis of "enzyme memory" (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982, 1983) was consequently rejected and we postulate that the observed protective effects of proteins can be explained by the respective bilirubin-binding characteristics of the proteins.

The glutathione S-transferase isoenzymes π and ρ similar or identical (Guthenberg & Mannervik, 1981). However, the kinetic mechanism of these isoenzymes has not been compared. The mechanism of action of isoenzyme π was being investigated in our laboratory (Ivanetich & Goold, 1989), and therefore an independent examination of isoenzyme ρ was undertaken. The kinetic mechanism of isoenzyme ρ was elucidated by a matrix of kinetic experiments which combined substrate concentration variation, product inhibition studies and computer modelling. Variation of the concentrations of the substrates CDNB and glutathione were consistent with either a random rapid equilibrium or ordered steady state mechanism. Product inhibition experiments confirmed a random BiBi rapid equilibrium mechanism with the formation of an enzyme-CDNB-product dead-end complex. This mechanism is identical to that of human placental

isoenzyme π, with slightly different evaluated kinetic constants (Ivanetich & Goold, 1989).

The microperoxidases, heme-peptides used to investigate heme protein function (Adams, 1990), have been shown to bind and inhibit isoenzyme π (Adams & Goold, 1990). A similar procedure was utilized to investigate the interactions of microperoxidases with isoenzyme ρ. Initially the procedure used for the preparation of the microperoxidases was investigated and substantially improved by various modifications. Subsequently, microperoxidases 8, 9 and 11 were shown to bind to and inhibit isoenzyme ρ in a time-dependent manner. Inhibition followed pseudo first-order kinetics with a half-life of approximately 900 seconds. The inhibition process was dependent on the peptide concentration and the apparent inhibition constant (K_i) increased with microperoxidase size from 0.57 to 1.62 and 3.15 μM for microperoxidases 8, 9 and 11, respectively. Binding to isoenzyme ρ was a monoexponential process with a calculated apparent dissociation constant (K_D) of 3.4×10^{-7} M. Glutathione did not affect MP-9 binding to isoenzyme ρ, but CDNB abolished microperoxidase binding and bilirubin increased the rate of binding. The rate of binding was similar for all three microperoxidases. These findings differ from those obtained with isoenzyme π (Adams & Goold, 1990) and we propose that the glutathione S-transferase ρ and π probably show small differences at the tertiary or topographical level, although they appear to be kinetically similar.

Contents.

	Page
Acknowledgements	ii
Abbreviations	iii
Publications	iv
Presentations	v
Summary	vi
List of Tables	xiii
List of Figures	xv

Chapter 1: Introduction. 1

1.1	Drug Metabolism	1
1.2	The Mercapturic Acid Pathway	1
1.3	The Glutathione S-Transferases	4
1.4	Occurrence of the GSH S-Transferases	5
1.5	Nomenclature and Classification	7
1.6	Purification of GSH S-Transferases	12
1.7	Substrates of the GSH S-Transferases	13
1.7.1	Xenobiotics	13
1.7.2	Endogenous Substrates	14
1.8	Glutathione: Properties and Reactions	18
1.9	The Chemical Mechanism of the GSH S-Transferases	19
1.10	The Kinetic Mechanism of the GSH S-Transferases	21
1.11	Reversible Binding and Inhibition	23
1.12	Irreversible Inhibition of the GSTs and Covalent Binding	25
1.13	The Conjugation of 1,2-Dibromoethane to GSH and Subsequent Toxicity of the Conjugate	27
1.14	The Characteristics of Bilirubin	29
1.15	Halothane: Historical Aspects	30
1.16	Halothane Metabolism	31
1.17	Halothane Hepatotoxicity	35
1.18	Additional Effects of Halothane	36
1.19	The Microperoxidases as Models of Heme Protein Function	37
1.20	Properties of the Microperoxidases	38
1.21	Microperoxidase Purification	39
1.22	Motivation for Study	40

Page

Chapter 2: Materials and Methods. 42

Materials 42
Chemicals 42
HPLC Columns 43

Methods 43
2.1 Assays for GST Enzyme Activity 43
2.1.1 Standard Assay Conditions 43
2.1.2 Cumene Hydroperoxide Assay 44
2.1.3 Centrifugal Analyzer 45
2.2 Protein Assays 46
2.2.1 Lowry Protein Assay 46
2.2.2 Bio-rad Protein Assay 47
2.2.3 Spectrophotometric Assays 47
2.3 Protein Gel Electrophoresis 48
2.3.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 48
2.3.2 Protein Staining 49
2.3.2.1 Coomassie Blue Stain 49
2.3.2.2 Bio-rad Silver Stain 49
2.4 Glutathione Assays 49
2.4.1 Glutathione Concentrations in the Millimolar Range 50
2.4.2 Glutathione Concentrations in the Micromolar Range 50
2.5 Anesthesia of Rats with Halothane 50
2.6 Purification of Rat Hepatic Isoenzymes 51
2.6.1 Cytosolic Preparation 51
2.6.2 Purification of the Mixture of Rat GST Isoenzymes 51
2.6.2.1 Method 1 51
2.6.2.2 Method 2 52
2.6.3 Preparation of Affinity Matrices 52
2.6.3.1 Preparation of S-Hexyl GSH 52
2.6.3.2 S-Hexyl GSH Sepharose 52
2.6.3.3 GSH-Sepharose 53
2.6.4 Separation of the Rat GST Isoenzymes by Chromatofocusing 53
2.6.5 Separation of GST Isoenzymes by Ion-Exchange Chromatography 54
2.7 Purification of Human Erythrocyte GSTs 55
2.8 Enzyme Incubations 56
2.8.1 Incubations with Inhibitors 56
2.8.2 Inhibitor Solutions 56
2.8.3 Bilirubin Incubations 57
2.8.4 Incubations with Microperoxidases 57
2.9 Reversible Activation/Inhibition by Halothane and DBE 57
2.9.1 Reversibility of Activation/Inhibition by Dilution 58

Page

2.10 Synthesis of S-(2,4-Dinitrophenyl)GSH 58
2.11 Preparation of Microperoxidases 58
2.11.1 Purification of Microperoxidases by HPLC 59
2.11.2 Extinction Coefficients 59
2.11.3 Binding of the Microperoxidases to GST q 60
2.12 Statistics and Calculations 60

Chapter 3: Halothane: Inhibition and Activation of Rat Hepatic Glutathione S-Transferases. 61

3.1 The Effect of Halothane Anesthesia on Rat Hepatic GST Activity <u>in vivo</u>. 61
3.2 Inhibition of GST Activity <u>in vitro</u> 63
3.3 Prevention of Halothane Inhibition by Cytosol <u>in vitro</u> 67
3.4 Reversal of Halothane Inhibition <u>in vitro</u> 68
3.5 Metabolism of Halothane and DBE <u>in vitro</u> 68
3.6 Reversible Activation by Halothane <u>in vitro</u> 69
3.7 Purification of Rat GSH S-Transferases 74
3.7.1 Effect of DTT on GST Activity 74
3.8 Effect of Solvents on Enzyme Activity 75
3.9 Linearity of GST Assays on Beckman Spectrophotometer 77
3.10 Linearity of Cumene Hydroperoxide Assay 78
3.11 Bromide Electrode 78
3.12 Standard Curves for GSH Assays 79

Chapter 4: The Reversible Inhibition and Activation of Rat Hepatic GST by 1,2-Dibromoethane <u>in vitro</u>. 80

Chapter 5: Bilirubin: Reversible Inhibition of GST 1-2 and Protection against Inhibition. 84

5.1 Time-Dependent Inhibition of GST 1-2 by Bilirubin 84
5.2 Reversal of Bilirubin Inhibition 85
5.3 Effect of Albumin on GST 1-2 Inhibition 85
5.4 Bilirubin Binding by Proteins 86
5.5 Protection against Bilirubin Inhibition by Proteins 89
5.6 Effect of Different Phosphate Buffers on Inhibition 90

Page

5.7 Alternative Methods used to Measure Free Bilirubin Concentrations 90
5.7.1 The Use of Ultrafiltration Membranes to Separate Albumin and Bilirubin 90
5.7.2 Use of Cation-Exchangers to Separate Protein and Bilirubin 91
5.7.3 Precipitation of Proteins with Organic Solvents and TCA 91
5.7.4 Bilirubin Extraction by Organic Solvents 92
5.7.5 Centrifugation of Bilirubin and Protein 92
5.7.6 Precipitation with Sulfuric Acid and Sodium Tungstate 92

Chapter 6: Kinetic mechanism of Erythrocyte GST ρ (rho). 93

6.1 Kinetic Mechanism of GST ρ 93
6.2 Product Inhibition Studies 100
6.3 Purification of Human Erythrocyte GST ρ (rho) 104
6.4 Linearity of the Multistat Centrifugal Analyzer 106

Chapter 7: Microperoxidases: Binding and Inhibition of GST ρ (rho). 107

7.1 Purification of Microperoxidases 107
7.2 Inhibition of GST ρ by Microperoxidases 110
7.3 Kinetics of the Binding of Microperoxidases to GST ρ 113
7.4 Concentration-Dependence of MP-8 Absorbance 118

Chapter 8: Discussion. 120

8.1 The Effects of Halothane on GST Activity 120
8.2 Reversible Activation and Inhibition of GST Activity by DBE 123
8.3 Inhibition of GST Activity by Bilirubin and Protection by Proteins 127
8.4 The Kinetic Mechanism of GST ρ (rho) 131
8.5 GST ρ and the Microperoxidases 135
8.6 Conclusions 138

References. 140

List of Tables.

		Page
1.1	Tissue and Species Distribution of Cytosolic GSH S-Transferases	6
1.2	Nomenclature of the Rat Cytosolic GSTs	8
1.3	Specific Activities of Rat and Human GSTs towards Various Substrates	9
1.4	Physicochemical Characteristics and Specific Activities with Various Substrates of Rat and Human Cytosolic GSTs	10
1.5	Classification of GST Isoenzymes	11
1.6	Specific Activities of Rat Cytosolic GSTs	14
1.7	Dissociation Constants for Non-Substrate Ligands Binding to Rat Soluble GSTs	23
2.1	Conditions for Spectrophotometric GST Assays	44
2.2	Conditions for the Preparation of MP-6, MP-8, MP-9 and MP-11	59
3.1	Effect of a Single Halothane Anesthesia on Rat Hepatic GST Activity *in vivo*	61
3.2	Effect of Multiple Halothane Anesthesias on Rat Hepatic GSH S-Transferase and GSH Peroxidase Activity	62
3.3	Time-Dependent Inhibition of the Mixture of Rat GSTs by Volatile Anesthetics and DBE *in vitro*	63
3.4	Time-Dependent Inhibition of Rat GST Isoenzymes by Halothane	66
3.5	Time-Dependent Inhibition of Human Placental GST π *in vitro*	66
3.6	Incubations of Cytosol with GST Inhibitors	67
3.7	Effect of Perfusion and Dialysis on the Time-Dependent Inhibition of Cytosolic GST Activity by Halothane	68
3.8	Effect of Different Solvents on GST Activity	76
3.9	Effect of Incubation with Acetone and Ethanol on GST Activity	76
5.1	Concentration-Dependence of Bilirubin Inhibition of GST 1-2	85
5.2	Effect of Bilirubin and Albumin on GST 1-2 Activity	86
5.3	Protection Against Bilirubin Inhibition of GST 1-2 by Proteins	89
5.4	Binding of Bilirubin and Proteins to Ion-Exchange Resins	91

		Page
6.1	Rate Equations for Bireactant Kinetic Mechanisms	98
6.2	Kinetic Parameters Obtained by Fitting the Initial Rate Data to Different Kinetic Mechanisms	99
6.3	Kinetic Parameters Obtained by Fitting Rate equations to Initial Rates in the Presence of Product Inhibition	103
7.1	Pseudo First-Order Rate Constants of Microperoxidase Binding to GST ρ and the Effects of Bilirubin and GSH Addition on the Rate constants	118
8.1	The Effect of Different values of α and β on the Type of Nonessential Activation	124
8.2	Interactions of DBE with rat GSTs	127
8.3	Kinetic Constants obtained for GST ρ and GST π with a Random Sequential BiBi Rapid Equilibrium Mechanism and Dead-End Complex Formation	135

List of Figures.

		Page
1.1	The Mercapturic Acid Pathway	3
1.2	The Reduction of Prostaglandin H_2 to Prostaglandin $F_{2\alpha}$ as Catalyzed by the GSH S-Transferases	15
1.3	The Reduction of Leukotriene A_4 Methyl Ester to Leukotriene C_4 Monomethyl Ester by Conjugation with GSH	16
1.4	The Enzymatic Isomerization of Δ^5-3-Ketosteroids to the corresponding α,β-Unsaturated Δ^4-3-Ketosteroids	17
1.5	The Structure of Reduced Glutathione	18
1.6	The Conjugation Reaction of GSH with CDNB Showing σ-Complex Formation	20
1.7	The Proposed Mechanism of Δ-Isomerase Activity	21
1.8	DBE Excretion and Activation Pathways	28
1.9	The Structure of Bilirubin IX-α Showing the Hydrogen Bonding between the Relevant Atoms	29
1.10	The Oxidative Metabolism of Halothane	32
1.11	The Reductive Metabolism of Halothane	33
1.12	The Amino Acid Sequence of the Various Microperoxidases Obtained by the Enzymatic Digestion of Cytochrome c	38
2.1	Diagrammatic Representation of the Multistat^R Centrifugal Analyzer Rotor	46
3.1	Time-Dependent Inhibition of GST Activity by Halothane	64
3.2	Concentration-Dependence of Halothane Inhibition	65
3.3	Metabolism of Halothane and DBE by Cytosolic GST in the Presence of GSH	69
3.4	Reversible Activation of Cytosolic GST Activity by Halothane with Respect to CDNB and GSH Variation	71
3.5	Reversible Activation of GST 1-2 by Halothane as a Function of CDNB Concentration	72
3.6	Reversible Activation of GST 1-1 and 3-3 by Halothane as a Function of CDNB Concentration	73
3.7	Time Course of GSH Dialysis	75
3.8	Linearity of CDNB Assay	77
3.9	Linearity of Cumene Hydroperoxide Assay	78
3.10	Linearity of Bromide Electrode	79
3.11	Concentration-Dependence of Millimolar GSH Assay	80
3.12	Concentration-Dependence of Micromolar GSH Assay	80

		Page
4.1	The Reversible Inhibition and Activation of Rat Cytosolic GST by DBE as a Function of CDNB and GSH Concentrations	81
4.2	Double Reciprocal Plots of Reversible Inhibition and Activation of GST 3-3 by DBE as a Function of CDNB and GSH Concentrations	82
4.3	Double Reciprocal Plots of Reversible Inhibition and Activation of Isoenzymes 1-2 and 4-4 as a Function of CDNB Concentrations	83
5.1	Time-Dependent Inhibition of GST 1-2 in the Presence of 4 μM and 8 μM Bilirubin	84
5.2	Concentrations of Free and Protein-Bound Bilirubin in Eluates from Sephadex G-25 Columns as a Function of Protein Concentration	88
6.1	Plots of Initial Rate Data with Curves Fitted to the Michaelis Menten Equation and Replots of the Fitted Kinetic Parameters	94
6.2	Double Reciprocal Plots of Initial Rates as a Function of GSH concentration in the Absence of Product	96
6.3	Initial Rates at Different GSH and Constant CDNB Concentrations	97
6.4	Double-Reciprocal lots of GST ρ Inhibition by S-(2,4-Dinitrophenyl)GSH as a Function of CDNB and GSH Variation	100
6.5	Replots of Kinetic Parameters Obtained with the Data in Fig. 6.4	101
6.6	Effect of Chloride on GST ρ Activity at Different Substrate Concentrations	102
7.1	HPLC Elution Profiles of Microperoxidases	108
7.2	Time-Dependent Trypsin Digestion of MP-11 at 40 °C, 30 °C, 20 °C and 10 °C	109
7.3	Arrhenius Plot of Data Shown in Fig. 7.2	109
7.4	Elution Profile on a Sephadex G-15 Column of a Cytochrome c Nagarse Digestion	110
7.5	Time-Dependent Inhibition of GST ρ by MP-8, MP-9 and MP-11	111
7.6	Inhibition of GST ρ by MP-8, MP-9 and MP-11	112
7.7	Absorbance Scans of the Time-Dependent Binding of MP-8 to GST ρ	113
7.8	Time-Dependent Binding of MP-9 to GST ρ	114
7.9	Plot of the Pseudo First-Order Rate Constants with Respect to MP-9 Concentration	115

		Page
7.10	Plot of The Calculated Absorbance Decrease of the Binding of MP-9 to GST ρ	116
7.11	The Binding of MP-9 to GST ρ in the Presence of Bilirubin	117
7.12	Absorbance of MP-8 as a Function of its Concentration	
8.1	Replots of Bilirubin Inhibition Experiments	128

Introduction

1.1 Drug Metabolism.

The diet of humans and animals consists not only of nutrients, but also contains foreign and non-nutritive compounds. The kidneys can excrete polar substances rapidly whereas lipid soluble materials, which includes many xenobiotics, are eliminated slowly and require prior metabolism to polar products. The pathways of drug metabolism catalyze various reactions, including oxidation, reduction, hydrolysis and conjugation. The enzymes involved in these pathway include the cytochromes P-450, monoamine oxidases, glucuronyl transferases, glutathione S-transferases* and enzymes of the mercapturic acid pathway, to name a few (Williams, 1959; Gillette, 1979). This thesis focuses on the glutathione S-transferases.

1.2 The Mercapturic Acid Pathway.

Although the significance of glutathione# (GSH; γ-glutamylcysteinylglycine) (Meister, 1988) in the detoxication of xenobiotics was recognized as early as 1936 (Boyland & Levi, 1936), the enzymes catalyzing GSH-dependent reactions were only discovered a quarter of a century later (Booth *et al*, 1960) when a hepatic soluble enzyme active in conjugating GSH with organic electrophiles to produce mercapturic acids (Booth *et al*, 1961), and an enzyme conjugating bromosulfophthalein (BSP) with GSH were discovered (Combes & Stakelum, 1961).
Subsequently, several proteins which bound ligands including bilirubin and bile acids (Levi *et al*, 1969), azocarcinogens (Ketterer *et al*, 1967) and cortisol metabolites (Morey & Litwack, 1969) were isolated. These proteins were later characterized as ligandin (Kaplowitz *et al*, 1973; Habig *et al*, 1974; Litwack *et al*, 1971) and as rat isoenzymes 1-1 and 1-2 (Jakoby *et al*, 1984). The proteins discovered by Booth *et al* (1960) and Combes and Stakelum (1961) have now been identified as GSTs (Mannervik, 1985).

---

* Footnote: In this thesis the glutathione S-transferases will also be referred to as the GSH S-transferases, transferases or GST.
\# Footnote: The terms GSH or glutathione apply to reduced glutathione; Oxidized glutathione is referred to as GSSG.

The GSH S-transferases catalyze the first step in the mercapturic acid pathway (Fig. 1.1), detoxifying potentially toxic electrophiles by converting them into hydrophilic compounds (Habig, 1983). Mercapturic acids are are end-products of the biosynthetic pathway proposed by Barnes *et al* (1959) and Bray *et al* (1959). The GSH thioethers produced by the GSTs are preferentially excreted into the bile and then the intestines and faeces, or reabsorbed into the intestines to undergo enterohepatic circulation (Nielsen & Rasmussen, 1977; Chasseaud, 1976). In the kidneys the conjugates are converted to L-cysteine derivatives by the γ-glutamyltranspeptidase and glycyl peptidase enzymes (Kozak & Tate, 1982), and are modified either to mercapturic acids by conjugation with acetyl CoA (Chasseaud, 1976) or converted to thiol derivatives, pyruvate and ammonia (Tateishi & Shimizu, 1980).

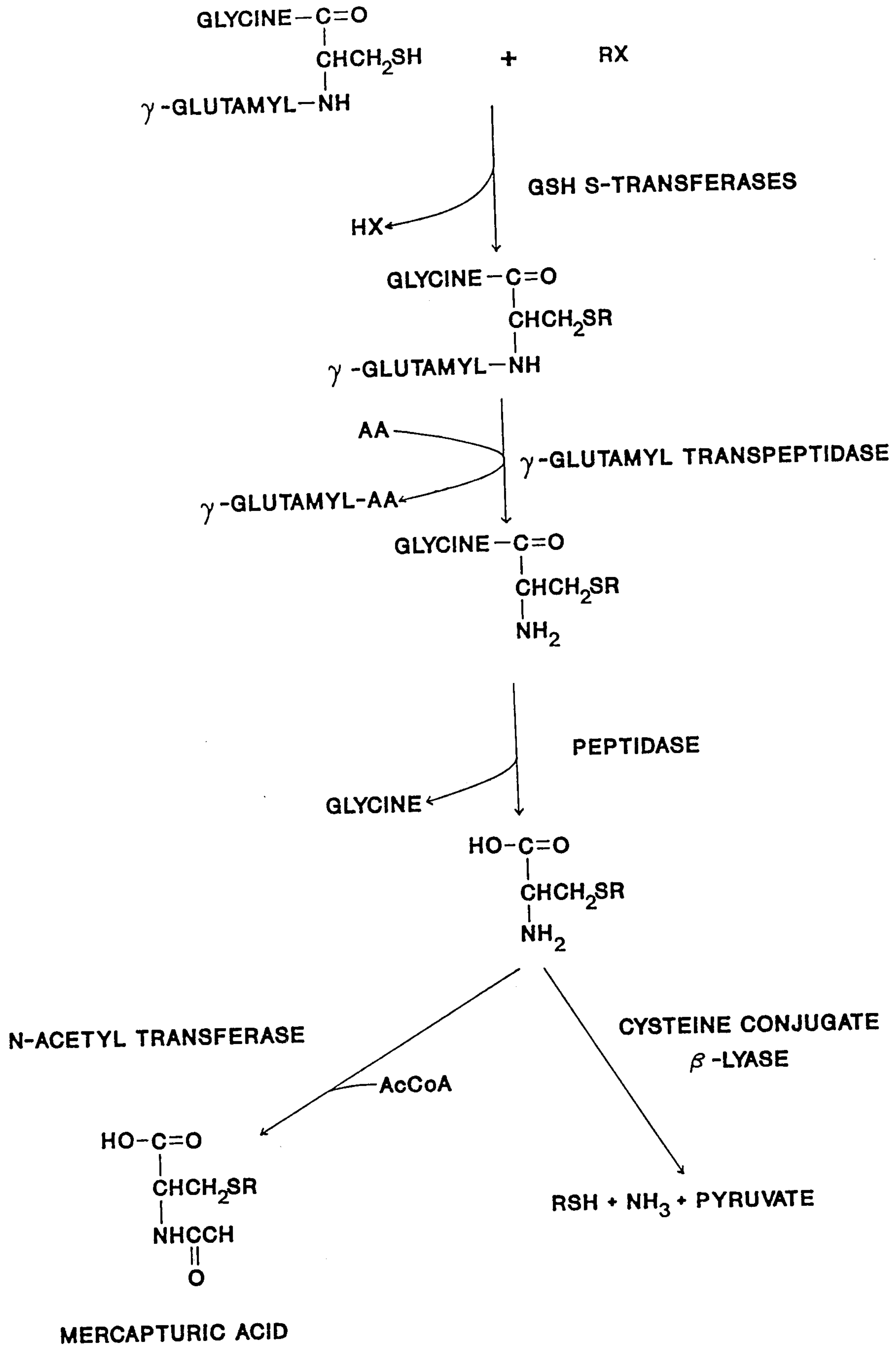


Fig. 1.1: The mercapturic acid pathway.
(adapted from Habig, 1983).

Bromobenzene, a potentially toxic xenobiotic, is metabolized by the enzymes of the mercapturic acid pathway (Habig, 1983). In the rat, bromobenzene is initially converted mainly to bromobenzene-3,4-epoxide by the cytochrome P-450 enzymes of the endoplasmic reticulum (Jollow *et al*, 1974). The epoxide is either enzymatically converted to a dihydrodiol by a microsomal epoxide hydrase enzyme (Daly *et al*, 1972), conjugated with GSH by cytosolic GSTs (Brodie *et al*, 1970; Habig, 1983) or non-enzymatically converted to 4-bromophenol (Daly *et al*, 1968; Gillette, 1979). Conjugation of the epoxide with GSH eventually produces bromophenylmercapturic acid as the end-product of the mercapturic acid pathway (Jerina *et al*, 1968; Boyland, 1971).

Other xenobiotics such as paracetamol (acetominophen) and naphthalene (Mitchell *et al*, 1974; Hayakawa *et al*, 1975) are also activated by cytochromes P-450 before being conjugated with GSH by the GSH S-transferases, while compounds such as chloronitrobenzenes, epoxides, α,β-unsaturated compounds, aflatoxin and aminoazo dye carcinogens are conjugated directly with GSH (Chasseaud, 1973; Boyland & Chasseaud, 1969; Ketterer *et al*, 1982). In animals, paracetamol dosage decreases hepatic GSH levels by more than 70% and cause liver necrosis (Mitchell *et al*, 1973; Potter *et al*, 1974). Toxic doses of bromobenzene decrease the mercapturic acid metabolite from 70% to 48% and increase the concentration of 4-bromophenol, which produces tissue necrosis (Zampaglione *et al*, 1973).

1.3 The Glutathione S-Transferases.

The glutathione S-transferases (EC 2.5.1.18) are a family of enzymes detoxifying a wide spectrum of xenobiotics by catalyzing their conjugation with GSH. This reaction is the first step of the mercapturic acid pathway (Habig, 1983), which is generally considered as a detoxication mechanism for the metabolism and excretion of mutagens, carcinogens and other toxic chemicals (Mannervik & Danielson, 1988). The GSTs are also active as GSH peroxidases and steroid isomerases, and thus catalyze a wide spectrum of reactions requiring GSH as co-substrate (Boyer & Kenney, 1985; Jakoby *et al*, 1976).
The GSTs also bind non-electrophilic substrates such as bilirubin and bile salts, and appear to act as intracellular transport or storage proteins. The GSTs can act as scavengers of alkylating agents and strong electrophiles by covalently binding

them, concomitantly inactivating the enzyme (Boyer & Kenney, 1985; Jakoby, 1978).

1.4 Occurrence of the GSH S-Transferases.

The GSTs are found in most aerobic organisms including mammals, insects, plants and bacteria (Table 1.1). With the exception of a membrane-bound microsomal form, the enzymes are cytosolic, water-soluble proteins (Morgenstern *et al*, 1984; Boyer, 1989). In mammalian tissues, concentrations are usually high (up to 10% of the total soluble protein), the GSTs being found in most organs and tissues (Table 1.1), although low levels of the enzyme are found in some non-mammalian species (Mannervik, 1985; Sugiyama *et al*, 1981).
The cytosolic GSTs usually occur as functional homo- or hetero-dimers. A trimeric microsomal form has been found in rats and humans, and a monomeric isoenzyme isolated from cell extracts of *Tetrahymena thermophila* (Boyer *et al*, 1986; Overbaugh *et al*, 1988). Numerous GST isoenzymes have been identified and isoenzyme expression is diverse as a result of variable or non-expression of isoenzymes (Suzuki *et al*, 1987), or multiple genes and hybridization of subunits (Mannervik, 1985). GST isoenzymes can be characterized by subunit composition, substrate specificity, binding affinity for non-substrate ligands, isoelectric point and inhibition characteristics (Boyer & Kenney, 1985; Mannervik & Danielson, 1988).

Table 1.1: Tissue and species distribution of cytosolic GSH S-transferases.

Species	Tissue	Reference
Rat	Brain	Senjo & Ischibashi, 1986
Rat	Erythrocytes	Dirr & Schabort, 1988
Rat	Heart	Tu *et al*, 1984
Rat	Hepatoma	Meyer *et al*, 1985
Rat	Kidney	Sheehan & Mantle, 1984
Rat	Liver	Habig *et al*, 1974
Rat	Lung	Guthenberg & Mannervik, 1979
Rat	Placenta	Di Ilio *et al*, 1986
Rat	Testis	Guthenberg *et al*, 1985
Human	Adrenal	Faulder *et al*, 1986
Human	Brain	Ogorochi *et al*, 1987
Human	Breast	Di Ilio *et al*, 1986
Human	Erythrocyte	Marcus *et al*, 1978
Human	Kidney	Tateoka *et al*, 1987
Human	Leukocytes	Seidegard *et al*, 1984
Human	Liver	Simons & Vander Jagt, 1977
Human	Lung	Dao *et al*, 1984
Human	Placenta	Awasthi *et al*, 1979
Human	Platelets	Federici *et al*, 1985
Human	Retina	Singh *et al*, 1984
Human	Spleen	Faulder *et al*, 1986
Human	Thyroid	Del Boccio *et al*, 1987
Human	Uterus	Di Ilio *et al*, 1988
Camel	Liver	Hunaiti & Sarhan, 1987
Dog	Liver	Wiener, 1986
Guinea pig	Liver	Hayakawa *et al*, 1977
Monkey	Liver	Hoesch & Boyer, 1988
Mouse	Liver	Warholm *et al*, 1986
Porcine	Liver	Grahnen & Sjoholm, 1977
Rabbit	Liver	Miyaura & Isono, 1986
Sheep	Liver	Reddy *et al*, 1983
Trout	Liver	Ramage & Nimmo, 1983
Shark	Liver	Sugiyama *et al*, 1981
House fly		Ohkawa *et al*, 1972
Mosquito		Hazelton & Lang, 1983
Mite		Mullin *et al*, 1982
Blue crab		Keeran & Lee, 1987
Flour beetle		Cohen, 1987
Pea		Williamson & Beverley, 1987
Corn		Mozer *et al*, 1983
Wheat		Williamson & Beverley, 1988
Galleria mellonella		Clark *et al*, 1977
Dirofilaria immititis		Jaffe & Lambert, 1986
Brugia pahangi		Jaffe & Lambert, 1986
Fusarium (Fungi)		Cohen *et al*, 1986
E. coli		Shishido, 1981
Issatchenkia orientalis		Tamaki *et al*, 1989

1.5 Nomenclature and Classification.

Early publications on the GSH S-transferases referred to them as aryl-, alkyl-, aralkyl-, alkene- or epoxide-transferases, depending on the structure of the electrophilic substrate (Boyland & Chasseud, 1969). However, several laboratories (Pabst *et al*, 1973; Askelöf *et al*, 1975) demonstrated the overlapping substrate specificities of the GSTs, thus necessitating an improved nomenclature (Jakoby *et al*, 1976). Various terminologies have been proposed for the rat GSTs and are briefly discussed below.

Habig *et al* (1974) designated the rat hepatic GSTs according to the reverse order of elution from CM-cellulose, *ie* isoenzymes D & E, C, B, A and AA. These proteins were dimers with subunits of approximately 25 000 daltons and could be differentiated by catalytic and immunological properties, although they exhibited overlapping substrate specificities (Habig *et al*, 1974, 1976). Ligandin, a protein thought to be important in the hepatic transport and storage of bilirubin, drugs and carcinogens (Litwack *et al*, 1971; Arias *et al*, 1976) was identified as isoenzyme B, with two distinct related subunits of 22 000 and 24 000 daltons. These subunits, designated Y_a and Y_c (Bass *et al*, 1977; Beale *et al*, 1982) also formed homodimers, *ie* Y_aY_a and Y_cY_c (Hayes & Clarkson, 1982). However, some laboratories referred to the Y_aY_a homodimer as ligandin (Jakoby *et al*, 1976). Isoenzymes A, C and D were subsequently shown to be composed of two different Y_b subunits and were renamed isoenzymes Y_bY_b, $Y_bY_{b'}$ and $Y_{b'}Y_{b'}$, respectively (Beale *et al*, 1983; Hayes, 1983).

As the nomenclature for the rat GSTs was clearly unacceptable and vague, a terminology* for the rat soluble GSTs denoting each distinct protein subunit by a Arabic numeral was recommended (Table 1.2) (Jakoby et al, 1984). This terminology reflects the subunit structure, heterogeneity and dimeric nature of the GSTs.

---

* Footnote: In this thesis the new nomenclature shown in table 1.2 will be used for the rat GST isoenzymes. Human GSTs are identified by Greek letters (Mannervik & Danielson, 1988). Erythrocyte GST isoenzymes will also be referred to as the acidic (ρ, rho) or basic erythrocyte GSTs.

Table 1.2: Nomenclature for the rat cytosolic GSTs.
(adapted from Jakoby *et al*, 1984, Mannervik & Danielson, 1988)

New Nomenclature	Previous Nomenclatures					
(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**	**(7)**
Isoenzyme 1-1	Ligandin	B	Ligandin	B_1	Y_aY_a	L_2
Isoenzyme 1-2	Ligandin	B	B	B_2	Y_aY_c	BL
Isoenzyme 2-2		AA		AA	Y_cY_c	B_2
Isoenzyme 3-3		A		A	Y_bY_b	A_2
Isoenzyme 3-4		C		C	$Y_bY_{b'}$	AC
Isoenzyme 4-4		D		D	$Y_{b'}Y_{b'}$	C_2
Isoenzyme 5-5		E		E		
Isoenzyme 6-6		MT			Y_aY_a	
Isoenzyme 7-7		P			Y_p or Y_f	
Isoenzyme 8-8		K			Y_kY_k	

(1) Jakoby *et al*, 1984; (2) Bass *et al*, 1977; (3) Habig *et al*, 1974; (4) Hayes *et al*, 1981; (5) Ketterer *et al*, 1983; (6) Bass *et al*, 1977, Ketterer *et al*, 1983; (7) Mannervik & Jensson, 1982.

GST isoenzymes containing subunits 1 and 2 display high isomerase and selenium-independent GSH peroxidase activity (Mannervik & Jensson, 1982), while isoenzymes with subunits 3 and 4 are reactive with DCNB and *trans*-4-phenyl-3-buten-2-one (Booth *et al*, 1961; Mannervik & Jensson, 1982). Epoxy propane is a good substrate for isoenzyme 5-5 (Fjellstedt *et al*, 1973) which does not bind S-hexyl GSH (Mannervik, 1985). (Table 1.3)

Table 1.3: Specific activities of rat and human GSTs towards various substrates. (adapted from Mannervik & Danielson, 1988)

	Specific activity (μmol/min/mg protein)								
Class	Alpha				Mu			Pi	
Isoenzyme[a]	1-1	2-2	B_1B_1	α-ε	3-3	4-4	μ	7-7	π
Substrate:									
CDNB[b]	50	17	82	64	58	17	187	24	105
DCNB	<0.04	<0.04	0.25	0.05	5.3	0.18	0.032	0.048	0.11
BSP	<0.01	<0.01	N.D.	0.005	0.94	0.04	<0.002	0.01	<0.002
T-4-P	<0.04	<0.04	N.D.	0.0015	0.05	1.18	0.36	0.22	0.01
Epoxy propane	<0.1	<0.1	0	0	0.53	1.37	0.11	N.D.	0.37
Cum. Hyd.	3.1	7.9	31	10.6	0.35	0.72	0.63	0.048	0.03
H_2O_2	<0.01	<0.01	N.D.	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
And. Dione	4.2	0.36	N.D.	8.0	0.02	0.002	0.12	N.D.	N.D.

[a] Isoenzymes with numerical designations refer to rat isoenzymes, Greek letters refer to human GSTs.
[b] Abbreviations: CDNB: 1-chloro-2,4-dinitrobenzene;
DCNB: 1,2-dichloro-4-dinitrobenzene; BSP: Bromosulfophthalein;
T-4-P: *trans*-4-phenyl-3-buten-2-one;
Epoxypropane: 1,2-epoxy-3-(ρ-nitrophenoxy)-propane;
Cum. Hydroperoxide: Cumene hydroperoxide; And. dione: Androstene dione.
N.D.: Not determined

The human GSTs are broadly classified into three groups according to their isoelectric points, namely the basic (pI = 7.8 - 8.9), near-neutral (pI = 6.0 - 6.5) and acidic (pI < 5) transferases (Kamisaka *et al*, 1981; Marcus *et al*, 1978; Guthenberg & Mannervik, 1981).
The human basic GSTs being characterized by high GSH peroxidase and isomerase activity (Warholm *et al*, 1983). Although five very similar basic isoenzymes (α, β, γ, δ, ε) were isolated from human liver (Kamisaka *et al*, 1975), some of these enzymes appear to be charged isomers of each other (Boyer & Kenney, 1985). These basic isoenzymes therefore cannot be rigorously identified and have collectively been specified as the "basic" transferases (Mannervik *et al*, 1983) or transferases "α-ε" (Warholm *et al*, 1983). A further three basic human GSTs, including two homodimers and a corresponding heterodimer, have also recently been characterized (Soma *et al*, 1986; Stockman *et al*, 1985, 1987).
The near-neutral isoenzymes are characterized by their relatively high activity with *trans*-4-phenyl-3-buten-2-one (Warholm *et al*, 1983) and are strongly inhibited by cibacron blue (Tahir *et al*, 1985). A near-neutral isoenzyme, μ, has been found at

concentrations of 15% - 20% of the total GST protein in approximately 60% of humans investigated (Mannervik, 1985; Boyer & Kenney, 1985).
Acidic GSTs have been found in the liver (ψ & ω) (Awasthi *et al*, 1980), placenta (π, pi) (Guthenberg *et al*, 1979; Polidoro *et al*, 1980) and erythrocytes (ρ, rho) (Marcus *et al*, 1978) Isoenzymes π and ρ are identical or closely related (Guthenberg & Mannervik, 1981).

Physicochemical characteristics of rat and human cytosolic GSTs reflect their similarity in size in spite of the wide spectrum of isoelectric points and overlapping substrate specificities (Table 1.4). These isoenzymes can be identified by substrate specificity, subunit molecular weight, isoelectric point, inhibition characteristics and immunological properties (Mannervik & Danielson, 1988).

Table 1.4: Physicochemical characteristics and specific activities with various substrates of rat and human cytosolic GSTs.
(adapted from Mannervik & Danielson, 1988)

Isoenzymes	Rat							Human			
	1-1	1-2	2-2	3-3	3-4	4-4	7-7	B_1B_1	B_2B_2	μ	π
Class	Alpha	Alpha	Alpha	Mu	Mu	Mu	Pi	Alpha	Alpha	Mu	Pi
Apparent subunit M.W.[a] (kD)	25	25/28	28	26.5	26.5	26.5	24	25	25	26.5	23
Subunit M.W.[b] (kD)	25434	N.D.[c]	25209	25806	N.D.	25592	23307	25526	N.D.	N.D.	N.D.
Amino acid residues/subunit	221	N.D.	220	217	N.D.	217	209	221	N.D.	N.D.	N.D.
Isoelectric point	10	9.9	9.8	8.9	8.0	6.9	7.0	8.9	8.4	6.6	4.8

[a] From SDS polyacrylamide gel electrophoresis.
[b] From the amino acid analysis.
[c] N.D.: Not Determined

Mammalian cytosolic GSTs have been classified into three groups, *viz* alpha, mu and pi, according to their physicochemical properties, *ie* substrate specificity, sensitivity to inhibitors, amino acid sequence and immunological cross-reactivity (Mannervik *et al*, 1985). Within each class, the isoenzymes have similar N-terminal amino acid sequences. Polyclonal antisera responsive to isoenzymes of one class do not cross-react with isoenzymes of other classes. The classes also exhibit differences in subunit molecular weights (on SDS-PAGE): Class pi

isoenzymes having the lowest apparent subunit molecular weights and class mu the highest (Table 1.5) (Mannervik *et al*, 1985).

Table 1.5: Classification of GST isoenzymes.
(Adapted from Mannervik *et al*, 1985).

	Class alpha	Class mu	Class pi
Members:	Rat 1-1 Rat 1-2 Rat 2-2 Human α-ε Mouse MI	Rat 3-3 Rat 3-4 Rat 4-4 Rat 3-6 Human μ Mouse MI	Rat 7-7 Human π Mouse MII
Specific substrates:	Cumene Hydroperoxide Androstene dione	BSP *trans*-4-phenyl-3-buten-2-one	Ethacrynic acid
Inhibitor sensitivities:	BSP Hematin	Cibacron blue Triphenyltin chloride	--

A distinct microsomal GST has been isolated from rats, mice and humans (Morgenstern *et al*, 1980, 1984; Andersson *et al*, 1988). The protein is a membrane-bound trimer with subunits of 14 000 daltons (Morgenstern *et al*, 1979; Boyer *et al*, 1986) and an isoelectric point at pH 10.1 (Morgenstern & DePierre, 1983). Unlike the cytosolic GSTs, the microsomal isoform is activated by sulfhydryl reagents such as N-ethylmaleimide (Morgenstern *et al*, 1979; Morgenstern & DePierre, 1983) or by washing the microsomes with liposomes (Boyer *et al*, 1986), although it is not induced by phenobarbital, 3-methylcholanthrene or *trans*-stilbene oxide (Morgenstern *et al*, 1980).

1.6 Purification of GSH S-Transferases.

GST purification is complicated by the multiplicity of isoenzymes. However, isolation has been greatly simplified by affinity chromatography, the most commonly used matrices containing S-hexyl GSH or GSH linked to epoxy-activated agarose.
S-hexyl GSH is coupled to agarose by the α-amino group of its glutamic acid residue (Guthenberg & Mannervik, 1981); GSH is linked to agarose through the cysteine thiol (Simons & Vander Jagt, 1977). The binding capacity of both matrices is equivalent (Mannervik, 1985), but there are differences: The S-hexyl GSH resin can bind glyoxalase 1 (Aronsson & Mannervik, 1977), but does not bind isoenzyme 5-5 under standard conditions (Mannervik, 1985). S-hexyl GSH is a GST inhibitor, although low concentrations are required for enzyme elution (Boyer & Kenney, 1985). In contrast, high concentrations of GSH are required for elution from the GSH matrix (Boyer & Kenney, 1985), and this method has been reported to alter the apparent isoelectric point of some GSTs (Ramage & Nimmo, 1983).

A survey of purification methods reported in the literature indicates that the most popular methods of GST isoenzyme separation are chromatofocusing and ion-exchange chromatography. Isoelectric focusing has been used to separate GST isoenzymes (Clark *et al*, 1973; Askelöf *et al*, 1975), but this method has limited capacity and is very slow (Mannervik, 1985). Chromatofocusing is a rapid method with higher capacity and has been used to purify rat and human GSTs (Mannervik & Jensson, 1982; Jensson *et al*, 1982; Vander Jagt *et al*, 1985). Fast protein liquid chromatography (FPLC) has also been used to purify rat and mouse liver isoenzymes by chromatofocusing (Ålin *et al*, 1985; Warholm *et al*, 1986).

Ion-exchange chromatography was originally used by Habig *et al* (1974) to separate rat hepatic isoenzymes. Cytosol was applied to DEAE-cellulose, which retained approximately 80% of the protein, and the GST isoenzymes were subsequently separated on CM-cellulose resin using a salt gradient, individual GSTs were further purified using hydroxylapatite (Habig *et al*, 1974). Other laboratories have used the same basic method with minor modifications, including affinity chromatography (Mannervik & Guthenberg, 1981; Pattinson, 1981), molecular sieving (Askelöf *et al*, 1975), and ion-exchange chromatography on TEAE cellulose (Boyer *et al*, 1983).

Various HPLC methods have been used in conjunction with affinity chromatography to identify and separate GSTs. All rat isoenzyme subunits can be identified and quantified by reverse-phase HPLC chromatography (Ostlund Farrants *et al*, 1987; Vandenberghe *et al*, 1988; Meyer *et al*, 1989), while anion-exchange HPLC has been used to separate charge isomers of human placental (Radulovic & Kulkarni, 1985, 1986) and erythrocyte GSTs (Singh *et al*, 1986).

1.7 Substrates of the GSH S-Transferases.

The GSTs catalyze a bimolecular reaction, usually between GSH and a electrophilic substrate. The enzymes display high affinity and specificity for GSH as nucleophilic substrate; In contrast, the binding of electrophilic compounds to the hydrophobic site is of low-affinity and broad-specificity (Keen *et al*, 1976). Catalysis appears to depend on: (a) the presence of a substrate sufficiently electrophilic to react with GSH; (b) the binding of both substrates (electrophile and GSH) to the enzyme; (c) the enhanced nucleophilicity of the GSH thiol group when bound to the enzyme (Kaplowitz, 1980). Among the electrophilic substrates of the GSTs are numerous xenobiotics, *eg* bromobenzene and paracetamol (Mitchell *et al*, 1974; Habig, 1983), but relatively few endogenous substrates. The latter include leukotrienes, prostaglandins, steroids and products of lipid peroxidation (Mannervik, 1985; Boyer, 1989). Several xenobiotic and endogenous GST substrates are discussed below:

1.7.1 Xenobiotics.

GST substrates include halo and nitro derivatives, epoxides, thio esters and α,β-unsaturated compounds (Habig *et al*, 1974). In addition to the substrates commonly used for identification and characterization of GSH conjugating activity, *eg* CDNB and DCNB, compounds conjugated with GSH include 1-menaphthyl sulfate (Gillham, 1973), 1,4-dihalobutanes (Marchand & Abdel-Monem, 1985), chlorotrifluoroethylene (Dohn & Anders, 1982), fluoroacetimide and 2,4-dinitrofluorobenzene (Kostyniak & Soiefer, 1984), propanolol (Sasame *et al*, 1987), styrene oxide (DePierre & Moron, 1979), bromosulfophthalein (Kaplowitz *et al*, 1973) and bromobenzene metabolites (Habig, 1983). The GSTs are stereoselective in the conjugation of some substrates, *eg* benz(α)pyrene

(Morgenstern *et al*, 1982), arene and alkene oxides (Dostal *et al*, 1987), aralkyl halides (Mangold & Abdel-Monem, 1980), stilbene oxide (deSmidt *et al*, 1987), and vicinal-dihaloalkanes (Livesey *et al*, 1982).
The GST isoenzymes display overlapping substrate specificities. CDNB is the most universal substrate and is often used to follow GST activity. Analysis of substrate specificity is frequently used for identification and characterization of GST isoenzymes (Boyer & Kenney, 1985). The substrate specificities of some rat isoenzymes are shown in Table 1.6.

Table 1.6: Specific activities of rat cytosolic GSTs.
(adapted from Mannervik & Danielson, 1988)

	Specific activity (μmol/min/mg protein)						
Class:	**Alpha**			**Mu**			**Pi**
Isoenzyme:	1-1	2-2	8-8	3-3	4-4	6-6	7-7
Substrate[a]:							
CDNB	50	17	10	58	17	190	24
DCNB	<0.04	<0.04	0.12	5.3	0.18	2.85	0.05
BSP	<0.01	<0.01	N.D.	0.94	0.04	N.D.	0.01
Ethacrynic acid	0.08	1.24	7.0	0.08	0.62	0.057	3.84
T-4-P	<0.01	<0.01	0.10	0.05	1.18	0.019	0.22
Epoxypropane	<0.1	<0.1	N.D.	0.53	1.37	N.D.	N.D.
C. Hydroperoxide	3.1	7.9	1.10	0.35	0.72	0.19	0.05
A. Dione	4.2	0.36	N.D.	0.02	0.002	N.D.	N.D.

[a] Abbreviations: CDNB: 1-chloro-2,4-dinitrobenzene;
DCNB: 1,2-dichloro-4-nitrobenzene; BSP: bromosulfophthalein;
T-4-P: *trans*-4-phenyl-3-buten-2-one;
Epoxypropane: 1,2-epoxy-3-(p-nitrophenoxy)propane;
C. Hydroperoxide: cumene hydroperoxide; A. Dione: androstene dione.
N.D.: Not determined

1.7.2 Endogenous substrates.

Few endogenous GST substrates have been identified. These include organic hydroperoxides, prostaglandin H_2, leukotriene A_4 and Δ^5-3-ketosteroids. One possible role of the enzyme in endogenous metabolism is the protection of lipid membranes from peroxidation. The peroxidase activity of the GSTs is not selenium-dependent (Lawrence & Burk, 1976; Prohaska & Ganther, 1977) and is catalyzed mainly by GST subunits 1, 2 and 5 (Mannervik & Danielson, 1988). These isoenzymes only display activity with organic hydroperoxides including fatty

acid peroxides and hydroperoxides (Lawrence & Burk, 1976; Prohaska & Ganther, 1977; Gibson *et al*, 1980; Tan *et al*, 1984; Rietjens *et al*, 1987; Williamson *et al*, 1986), and products of lipid peroxidation such as methyl linoleate ozonides, cholesterol α-oxide and malondialdehyde (Vos *et al*, 1987; Meyer & Ketterer, 1982; Burk *et al*, 1980).

The selenium-independent GSH peroxidases, *ie* the GSTs, also catalyze the reduction of prostaglandin H_2 to prostaglandin $F_{2\alpha}$ by GSH (Fig. 1.2) (Burgess *et al*, 1987; Hong *et al*, 1989). The reaction is catalyzed in rats predominantly by isoenzymes containing subunits 1 and 2 (Chang *et al*, 1987) and in sheep lung by GSTs with subunits of the same size as rat subunit 1 (Hong *et al*, 1989). Selenium-dependent GSH peroxidases catalyze the formation of prostaglandin H_2 from prostaglandin G_2, the selenium-independent and selenium-dependent GSH peroxidases therefore interacting in the biosynthesis of prostaglandin $F_{2\alpha}$ (Burgess *et al*, 1987; Hong *et al*, 1989).

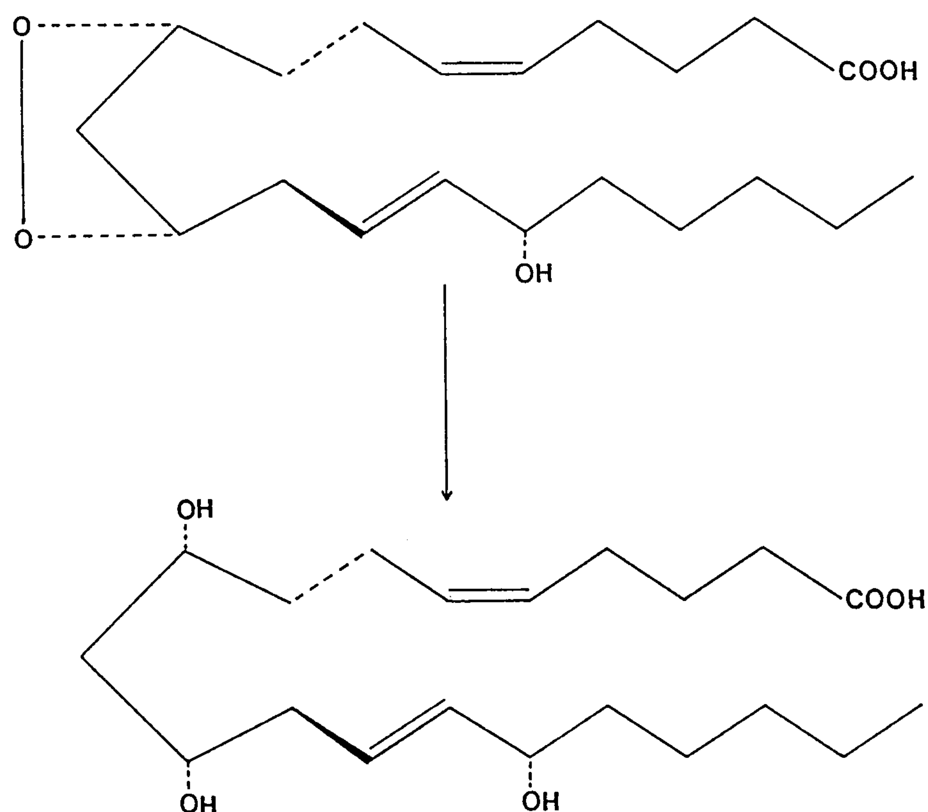


Fig. 1.2: The reduction of prostaglandin H_2 to prostaglandin $F_{2\alpha}$ as catalyzed by the GSH S-transferases.
(adapted from Hong et al, 1989)

The metabolism of leukotriene A_4 to leukotriene C_4 (Fig. 1.3) is catalyzed by both rat and human GSTs, with the rat 4-4 and human near-neutral (μ) isoenzymes

being the most active (Chang *et al*, 1987; Söderström *et al*, 1985; Mannervik *et al*, 1984). However, a unique microsomal enzyme is primarily responsible for the reduction of leukotriene A_4 to leukotriene C_4 *in vivo* (Söderström *et al*, 1988; Yoshimoto *et al*, 1985).

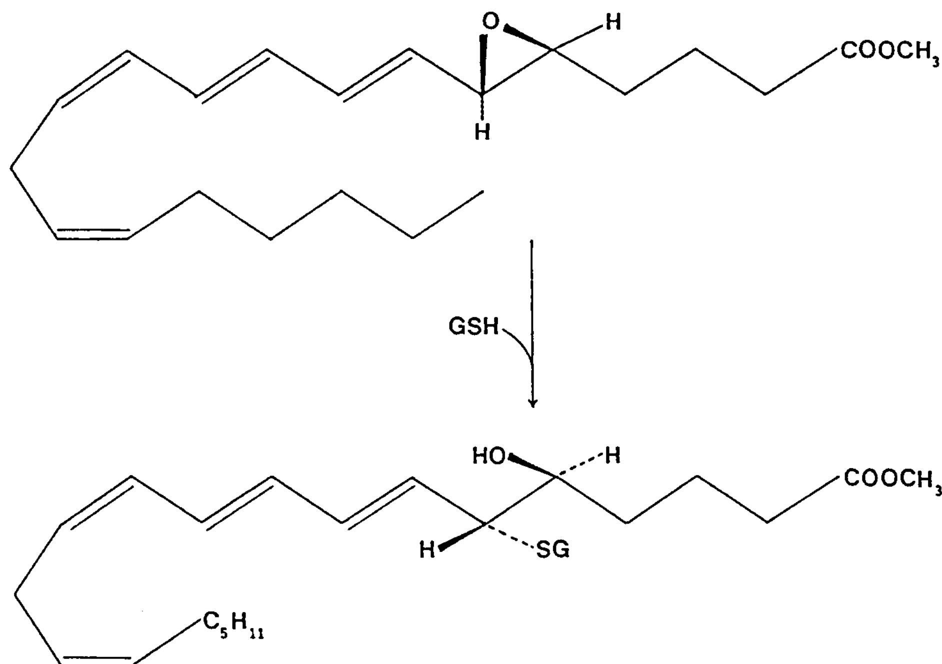


Fig. 1.3: The reduction of leukotriene A_4 methyl ester to leukotriene C_4 monomethyl ester by conjugation with GSH as catalyzed by cytosolic GST. (adapted from Mannervik et al, 1984).

Principally associated with Δ^5-3-ketosteroid isomerase activity is isoenzyme 1-1 (Mannervik & Jensson, 1982), catalyzing the isomerization of Δ^5-3-ketosteroids to the corresponding Δ^4-3-ketosteroids (Fig. 1.4). These enzymes are activated by GSH, which decreases the K_m for Δ^5-androstene-3,17-dione, although GSH is not consumed in the reaction. This is a unique GST reaction as GSH is not expended (Benson & Talalay, 1976).

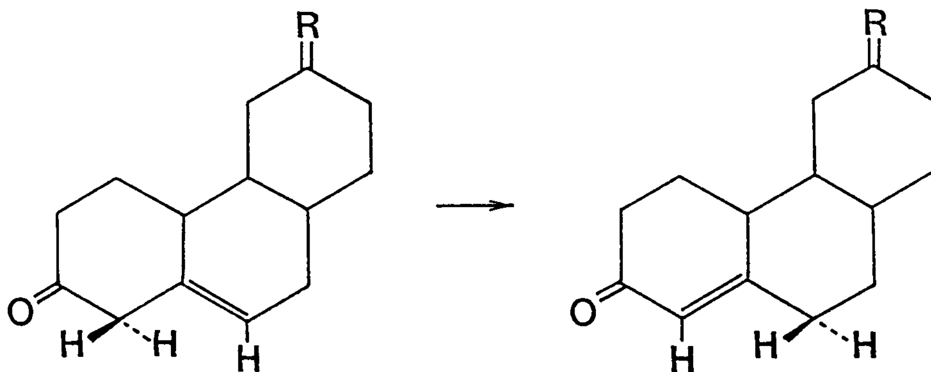


Fig. 1.4: The enzymatic conversion of Δ^5-3-ketosteroids to the corresponding α,β-unsaturated Δ^4-3-ketosteroids.
R = O or α-H, β-CH_3CO
(adapted from Benson et al, **1977)**

Furthermore, products of lipid peroxidation comprising 4-hydroxynonenal, 4-hydroxydecenal (Ålin *et al*, 1985), cholesterol α-oxide (Meyer & Ketterer, 1982), methyl linoleate ozonides (Vos *et al*, 1987) are endogenous GST substrates. Isoenzyme 4-4 exhibits the highest activity with 4-hydroxynonenal and 4-hydroxydecenal (Ålin *et al*, 1985).

Recently two fatty acid ethyl ester synthases, which metabolize alcohol in a non-oxidative process, have been proposed to be unidentified human neutral and acidic GSTs (Bora *et al*, 1989a, 1989b).

1.8 Glutathione: Properties and Reactions.

Glutathione is the most prevalent cellular thiol and typically present at concentrations of 0.1 to 10 mM (Meister, 1988; Fahey, 1977). Characteristic features are a γ-glutamyl linkage and sulfhydryl (-SH) group (Meister, 1988).

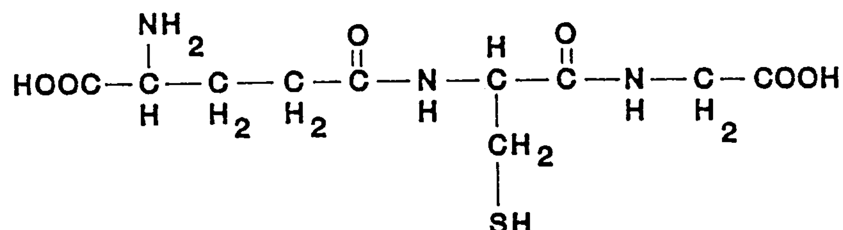


Fig. 1.5: The structure of reduced glutathione. (adapted from Meister, 1988).

The sulfhydryl group is a strong nucleophile, and can function as a reducing agent for disulfides and peroxides; react with aldehydes to yield thio-hemiacetals; and form conjugates with electrophiles (Ketterer, 1982). GSH protects cells against radiation damage, oxygen toxicity and toxic compounds. GSH is required for leukotriene, prostaglandin and ketosteroid synthesis, and is a substrate for the GSH peroxidases, GSH transhydrogenases (thiol transferases) and GSTs (Meister, 1988; Meister & Anderson, 1983).

GSH can react with the electrophilic centers of substrates by a variety of reactions, *ie* Michael additions, reactions with electrophilic cations, or nucleophilic attack involving the opening of a strained ring system, as well as nucleophilic displacement at either a saturated or aromatic carbon atom (Ketterer, 1982). The reaction of GSH with electrophilic substrates depends on the chemical hardness or softness of the electrophile. Soft electrophiles (*eg* polarized double bonds) have a readily polarizable low positive charge density at the electrophilic centre whereas hard electrophiles have a large positive charge density. The chemical selectivity of nucleophiles reacting with electrophiles is based on the high potential energy barrier to reactions of hard electrophiles with soft nucleophiles, and *vice versa*, resulting from the high energy of the transition state (Coles *et al*, 1985).

The large atomic volume and diffuse electron charge density of sulfur make thiol groups particularly soft nucleophiles (Pearson & Songstad, 1967). The reaction of hard electrophiles with soft nucleophiles is slow and GSH will therefore react more rapidly, and often non-enzymatically, with soft electrophiles, whereas the conjugation of hard electrophiles with GSH is unfavorable and requires a

enzymatic reaction to proceed at a reasonable rate (Ketterer, 1986; Ketterer *et al*, 1983).

Although the GSTs are relatively specific for GSH as the nucleophile (Jakoby, 1978) other thiol compounds such as propylthiouracil (Yamada & Kaplowitz, 1980), peptide analogues of GSH (Adang *et al*, 1988, 1989) and β-mercaptoethanol (Principato *et al*, 1988) can substitute for GSH in the enzymic conjugation reaction. Observations with GSH peptide analogues have shown that either the GSH-binding sites of different isoenzymes were not identical, or conformational states induced by binding of the analogues could affect catalytic ability (Adang *et al*, 1989). The requirement of S-methylglutathione for the conjugation of β-mercaptoethanol with CDNB by isoenzyme 4-4 was thought to indicate a dual role for GSH in the catalytic reaction of GSTs, *ie* to induce a catalytically efficient conformation of the enzyme and to provide the sulfhydryl group required as a substrate for the reaction (Principato *et al*, 1988).

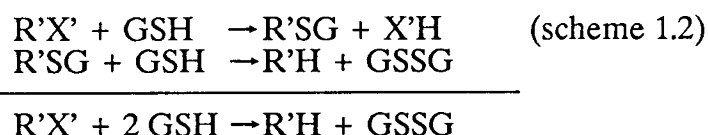
1.9 The Chemical Mechanism of the GSH S-Transferases.

The GSTs catalyze two types of reactions (Douglas, 1987):

(1) The formation of a stable GSH conjugate by nucleophilic attack of GSH at an electrophilic centre in the second substrate (scheme 1.1). This reaction takes place with most of the commonly used GST substrates, including CDNB and DCNB.

$$RX + GSH \rightarrow RSG + XH \qquad \text{(scheme 1.1)}$$

(2) In the second class of reaction, as exemplified by the peroxidase activity of the GSTs (scheme 1.2), two reduced products and GSSG are produced. The enzymic reaction forms an unstable intermediate (R'SG), the latter is then non-enzymatically conjugated with a further molecule of GSH to generate a reduced product (R'H) and GSSG. Examples of R'X' are the organic nitrates and organic hydroperoxides.



$$\begin{array}{l} R'X' + GSH \rightarrow R'SG + X'H \qquad \text{(scheme 1.2)} \\ R'SG + GSH \rightarrow R'H + GSSG \\ \hline R'X' + 2\,GSH \rightarrow R'H + GSSG \end{array}$$

Hammett plots of the enzyme-catalyzed conjugation of 4-substituted 1-chloro-2-nitrobenzenes with GSH demonstrated the nucleophilic nature of the reaction and the strong dependence on the electrophilicity of the non-thiol

substrate (Keen *et al*, 1976). The rate enhancement observed with GST 3-4 for the conjugation of CDNB with GSH at pH 7.0 was approximately 500 M (Jencks, 1969), denoting that the GSTs are not powerful catalysts (Jakoby, 1978).

Investigation of the chemical mechanism of isoenzyme 4-4 with several GSH peptide analogs and 4-substituted 1-chloro-2-nitrobenzenes has shown that the GSTS reduce the pK_A of enzyme-bound GSH by 2 to 2.5 pK units to a pK_A of approximately 6.6 (Chen *et al*, 1988; Jung *et al*, 1972; Reuben & Bruice, 1976). With site occupancy data this was consistent with the presence of either a GSH thiolate ($E.GS^-$) or ion-paired thiolate ($EH^+.GS^-$) at the active site (Graminski *et al*, 1989a). Using substrate analogues it was resolved that the formation of a σ-complex was the rate-determining step for the GSH-conjugating reaction of the GSTs (Chen *et al*, 1988). The pH-dependence of the apparent σ-complex formation constants implied a double-ionization mechanism (*eg* $H^+E.GSH \rightleftharpoons H^+E.GS^- \rightleftharpoons E.GS^-$) with pK_A values of 5.7 and 7.6 for the thiol and a protonated base at the active site (Graminski *et al*, 1989b).

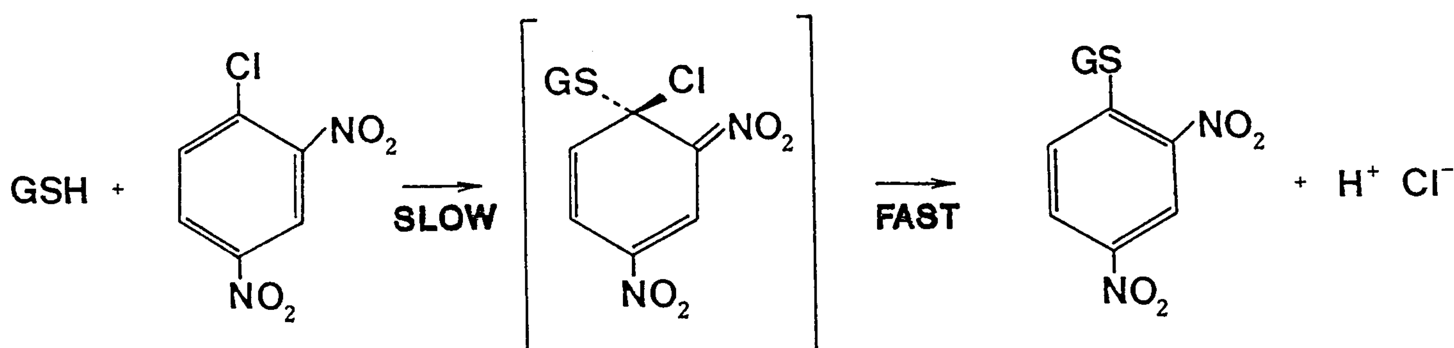


Fig. 1.6: The conjugation reaction of GSH with CDNB showing σ-complex formation.
(adapted from Graminski et al, 1989, Boyer & Kenney, 1985).

The steroid isomerase activity demonstrated by some of the GST isoenzymes (Benson & Talalay, 1976) catalyzing the conversion of Δ^5-steroids to Δ^4-steroids involves the rearrangement of a double bond coincident with a 1,3 proton shift (Spector, 1982). Transfer of the hydrogen from atom C-4β to C-6β involves the partial exchange of the proton with the medium (Talalay & Wang, 1955; Smith *et al*, 1980) and is stereospecific and intramolecular (Malhotra & Ringold, 1965; Viger *et al*, 1981). The stereochemistry of the reaction provides evidence for a single basic group at the active site (Wang *et al*, 1963; Malhotra & Ringold, 1965; Viger *et al*, 1981; Hanson & Rose, 1975), with possibly an electrophilic or

proton-donating group at the active centre polarizing the C-3 carbonyl group (Spector, 1982).

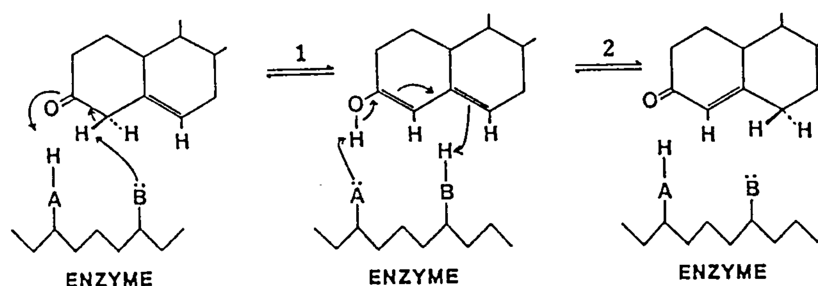


Fig. 1.7: The proposed mechanism of Δ-isomerase activity.
(1). Protonation of carbonyl, loss of 4*β*-proton.
(2). Reprotonation at 6*β* carbon, deprotonation of carbonyl.
A = proton donor, B = proton acceptor
(adapted from Spector, 1982, Malhotra & Ringold, 1965)

1.10 The Kinetic Mechanism of the GSH S-Transferases.

The detailed kinetic mechanism of the GSTs has been elucidated for only a small number of isoenzymes. A rapid equilibrium or steady state random sequential mechanism appears to be favored at present for all isoenzymes studied.

The kinetic mechanism of GST 3-3 has been investigated most intensively and displays non-Michaelis-Menten kinetics. Initial rate, product inhibition and binding studies reflect a substrate-dependent biphasic kinetic mechanism (Pabst *et al*, 1974; Mannervik, 1985). The data were initially thought to reflect two different enzymic pathways as determined by the GSH concentration: At low GSH concentrations, the electrophilic substrate was proposed to bind first in a ping pong mechanism, and at high GSH concentrations the electrophile bound after GSH in an ordered sequential mechanism (Pabst *et al*, 1974). Analysis by non-linear regression suggested that the rate equation required a constant term in the denominator, thus excluding a ping pong pathway and providing evidence for a random sequential mechanism (Mannervik & Askelöf, 1975). Evidence for a random sequential pathway has subsequently been provided by stereochemical studies with phenethyl halides (Mangold & Abdel-Monem, 1980, 1983), and experiments with a spin-labelled product (Schramm *et al*, 1984) and GSH analogues (Chen *et al*, 1988).
Building upon the simple random steady state mechanism, Jakobson *et al* (1977) investigated possible contributions from enzyme-product complexes. They

concluded that a steady state random sequential mechanism with kinetically significant enzyme-product complexes was favored over simple mechanisms, even though the rate equation contained redundant parameters when analyzed by regression methods (Jakobson *et al*, 1977, 1979a). However, it has subsequently been argued that the simplest steady state mechanism without product inhibition is sufficient to explain the observed kinetics of isoenzyme 3-3. This conclusion was based on the observation that the random sequential BiBi steady state mechanism was the simplest mechanism consistent with the observed kinetics, and that more complex models were overdetermined (Ivanetich *et al*, 1990).
The anomalous behavior of GST 3-3 cannot be explained by cooperativity between enzyme subunits or by the effects of ethanol, used as a solvent for the electrophilic substrate (Jakobson *et al*, 1977, 1979b). Possible conformational changes induced by the binding of GSH to the protein have been proposed as an explanation of the non-hyperbolic kinetics observed with some of the GSTs (Principato *et al*, 1988).

The kinetic mechanism of GSTs appears to be isoenzyme-specific since the human placental isoenzyme π, the major bovine brain GST and GST 1-1 exhibit a rapid equilibrium random sequential mechanism, as characterized by initial rate and product inhibition experiments (Ivanetich & Goold, 1989; Young & Briedis, 1989; Schramm *et al*, 1984).

1.11 Reversible Binding and Inhibition.

The GSTs reversibly bind ligands such as heme, bilirubin, steroids, bile acids and other amphipathic and hydrophobic molecules. Association constants for ligandin range from 2×10^5 to 10^8 M^{-1}. These ligands are in general not metabolized by the enzyme, hence the term "non-substrate" ligands (Jakoby, 1978). Although all the GSTs bind non-substrate ligands, the affinity for these ligands is variable, as shown in Table 1.7.

Table 1.7: Dissociation constants for non-substrate ligands binding to rat soluble GSTs.
(adapted from Boyer & Kenney, 1985)[a]

	K_D (μM) Ligand		
	Bilirubin	Hematin	Indocyanine green
Isoenzyme:			
1-1	0.34		0.55
1-2	0.68 (2)	(0.1)	0.94 (3)
2-2	4.1 (100)	(4)	3.69 (100)
3-3	(15)	(2)	(3)
3-4	(3)	(7)	(1)

[a] Dissociation constants were obtained from Boyer *et al*, 1983, values in parenthesis are from Ketley *et al*, 1975, and Arias *et al*, 1976.

One of the primary *in vivo* ligands for the GSTs is bilirubin, which usually binds to rat GSTs at both high and low affinity sites, the latter acting to decrease enzyme activity.
Human hepatic ligandin has no high-affinity bilirubin-binding sites, whereas rat isoenzymes 1-2 and 3-3 contain primary and secondary bilirubin binding sites (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982). Although bilirubin binding does not affect the catalytic activity of human placental GST π (Vander Jagt *et al*, 1981), activity of human hepatic ligandin and rat GSTs 1-2 and 3-3 is decreased by bilirubin (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982, 1983).
Isoenzymes containing subunits 1 and 2 (1-1, 1-2, 2-2) appear to bind bilirubin at a single site (Maruyama *et al*, 1984; Ketley *et al*, 1975). Covalent labelling of GST 1-1 and 2-2 with a bilirubin enol ester derivative showed that equimolar ratios of ester and enzyme blocked ligand binding, but did not affect enzyme activity. It was also suggested that a single high-affinity bilirubin binding site is formed on

interaction of the subunits and does not reside on a particular subunit (Boyer, 1986).

In some instances, inhibition of GST catalytic activity by non-substrate ligands is pH-dependent, although binding is not. Indocyanine green, biliverdin and chenodeoxycholate inhibit isoenzymes 1-2 and 2-2 at pH 6.0, but not at pH 8.0, although indocyanine green and biliverdin bind to the enzymes at both pH 6 and pH 8. The proteins were therefore presumed to form catalytically active enzyme-inhibitor-substrate complexes at the high pH and behave as both enzymes and binding proteins (Boyer *et al*, 1984).

The formation of kinetically stable conformational isomers, which are dependent on the order of addition of reactants and pH, has been postulated to take place on binding of bilirubin to GST (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982). The addition of GST to incubation mixtures containing foreign proteins and bilirubin suggested that the protein allowed the retention of GST activity. However, the concentrations of foreign proteins required for this effect was variable (Vander Jagt *et al*, 1983). It was proposed that bilirubin has two roles: It must first bind to the enzyme to initiate conformational changes, subsequent to which bilirubin competes with the foreign protein to establish the GST conformations formed. It was concluded that weak protein-protein interactions take place between the foreign protein and the bilirubin-GST complex. The ability of foreign proteins to regulate the conformational states of the GSTs was proposed to represent a type of enzyme memory, since the different conformational states were apparently stable (Vander Jagt *et al*, 1983).

The main driving force for the binding of non-substrate ligands to GSTs is hydrophobic in nature (Tipping *et al*, 1976). For example, the tendency of porphyrins to aggregate is paralleled by increased binding affinities of porphyrins for the GSTs (Tipping *et al*, 1976) Also, conjugation of non-polar ligands with sulfate or GSH only increased binding affinity to a small extent, illustrating that apolar binding dominated in the binding process (Tipping *et al*, 1976). The secondary binding site is possibly charged to facilitate binding, as indicated by the inhibitory effect of different porphyrins and bile acids (Smith *et al*, 1985; Boyer *et al*, 1984). Comparison of inhibition by chenodeoxycholate, lithocholate and cholate showed that the binding site was relatively hydrophobic with a significant ionic interaction at the binding domain (Hayes & Mantle, 1986).

The role of the GSTs in the intracellular transport and/or storage of ligands has been difficult to determine, and it has been suggested that these enzymes act as intracellular binding proteins, thereby increasing cytosolic ligand concentrations (Boyer, 1989; Tipping & Ketterer, 1981; Jakoby 1978).
Evidence for the role of the GSTs in the hepatic uptake of organic anions is provided by the following observations:
(1.) Several species of amphibia have no GST (ligandin) before metamorphisis and show delayed plasma disappearance of injected BSP and/or bilirubin; After metamorphisis these animals have abundant GST and show selective hepatic uptake of BSP and bilirubin (Levine *et al*, 1971).
(2.) Newborn guinea pig, rat, monkey and humans exhibit slow plasma clearance of, and/or low hepatic content of organic anions. This process matures in the first 10 days after birth, in parallel with increased hepatic GST (ligandin) content (Levi *et al*, 1970; Arias, 1970).
(3.) Induction of ligandin is associated with increased disappearance of organic anions from the plasma (Reyes *et al*, 1971).
(4.) PB-induction of dogs reduced the efflux of bilirubin from the liver, but did not change hepatic bilirubin influx (Arias *et al*, 1976).
(5.) Administration of bilirubin to normal rats decreased the transfer of triiodothyronine, a high-affinity GST ligand, from the plasma into the liver (Lichter *et al*, 1976).
(6.) Decreased BSP excretion and GST levels were determined after partial hepatectomy (Oleaga *et al*, 1987); Increased induced intracellular ligandin concentrations enhanced the net hepatic uptake of bilirubin (Wolkoff *et al*, 1979).
(7.) A case of Rotor's syndrome, a condition of familial hyperbilirubinemia with prolonged plasma BSP retention has been reported in which normal GST activity was significantly decreased (Adachi & Yamamoto, 1987).

1.12 Irreversible Inhibition of the GSTs and Covalent Binding.

A wide range of compounds covalently bind to the GSTs and inhibit enzyme activity. As with reversible binding, covalent inhibition of the GSTs is also isoenzyme-dependent. It has been predicted that strong electrophiles will react with the nucleophilic groups of proteins with which they come into contact to form covalent bonds. Thus, considering the high concentrations of the GST enzymes, this type of interaction is expected. This process usually leads to the loss of GST activity (Jakoby, 1978).

Several GST substrates are inhibitors of GSH conjugating activity. CDNB was first reported to inhibit GST activity by Pabst *et al* (1974). The human lung acidic isoenzyme is inhibited in a pseudo first-order process by CDNB, which bound to all human lung isoenzymes (Corrigal *et al*, 1989). Inactivation of GST π by CDNB, MP-8 and buffer has been proposed to result from solvation of the hydrophobic active centre (Adams *et al*, 1989); A second mechanism of inhibition appeared to involve the possible covalent binding of CDNB to a sulfhydryl group near the catalytic site (Adams & Sikakana, 1990). Ethacrynic acid also inhibits GST activity (Ahokas *et al*, 1984), covalently binding to isoenzyme 3-4 (Yamada & Kaplowitz, 1980).
Several conjugates and analogs of GSH are inhibitors of GST activity. The GSH analogs γ-L-Glu-SerGly and γ-L-Glu-AlaGly are dead-end inhibitors of isoenzymes 3-3 and 4-4 (Chen *et al*, 1985). S-hexyl, S-benzyl and S-(2,4-dinitrophenyl) GSH conjugates are strong, moderate and weak inhibitors, respectively, of isoenzymes 1-1, 2-2, 3-3 and 3-4, but have no effect on isoenzymes 4-4 and 5-5 (Ong & Clark, 1986).

Other covalent inhibitors of GST activity include carcinogens, drugs as well as spectrum of other widely used chemicals. In this respect binding to the GSTs is important in the detoxification of carcinogenic aminoazo dyes, polycyclic aromatic hydrocarbons and aromatic amines (Smith *et al*, 1977). Industrial solvents and chemicals such as chloroform, carbon tetrachloride, bromobenzene and 1,2-dibromoethane also preferentially inhibit certain GST isoenzymes (Aniya & Anders, 1985a; Aniya & Anders, 1985b; Ivanetich *et al*, 1984; Botti *et al*, 1982). Other GST inhibitors include cuprous complexes, mercury and cadmium (Dierickx, 1982, 1986), penicillin and cephalosporins (Polidoro *et al*, 1984) and paracetamol metabolites (Wendel & Cikryt, 1981).

1.13 The Conjugation of 1,2-Dibromoethane to GSH and Subsequent Toxicity of the Conjugate.

1,2-Dibromoethane (ethylene dibromide; DBE) is a colorless, dense, non-flammable liquid with a distinctive chloroform-like odor. DBE is used to fumigate vegetables, fruits and grains, and a lead scavenger in petrol (Rannug, 1980; USEPA, 1977). Approximately 2.5 million kg were used in the U.S.A. in 1975 and low concentrations of DBE have been detected in fumigated foods and ambient air (Fishbein, 1979).

Exposure of animals to DBE causes early mortality, weight loss, tumor formation and influences reproduction; DBE is a strong mutagen in microorganisms (Bogdan & Grey, 1984; Weisburger, 1985; Rannug, 1980; Wong *et al*, 1982). Although the effect of industrial exposure to DBE on reproduction and fertility is contentious (Weisburger, 1985; Ratcliffe *et al*, 1987), acute exposure has caused death (Weisburger, 1985). In monkeys the location of tissue binding corresponds to tissue lesions observed in DBE-poisoned humans (Brandt *et al*, 1987). In mice, renal and hepatic non-protein sulfhydryl concentrations were decreased by DBE (Kluwe *et al*, 1981), while DNA synthesis, mitotic activity (Ledda-Columbano *et al*, 1987) and DNA damage were increased (Kitchin & Brown, 1986). DBE adducts bound to DNA, RNA and proteins in all major types of rat tissue (Nachtomi & Sarma, 1977; Hill *et al*, 1978).

DBE toxicity is probably mediated by two separate mechanisms, *viz* oxidative metabolism and conjugation with GSH (van Bladeren *et al*, 1980). Treatment of hepatocytes with DBE decreases GSH levels and increases covalent binding to cellular macromolecules (Albano *et al*, 1984). Thirty to forty percent of a single dose of DBE administered to was excreted as the mercapturic acid (van Bladeren *et al*, 1980). Isolated metabolites include N-acetyl-S-(β-hydroxyethyl) cysteine, S-(β-hydroxyethyl) cysteine, S-(β-hydroxyethyl) GSH and S-(β-hydroxyethyl) mercapturic acid (Nachtomi *et al*, 1966; Nachtomi, 1970; Edwards *et al*, 1970). DBE reacts efficiently with sulfhydryl groups *in vitro* (Edwards *et al*, 1970) and GSH conjugation may be important in the mutagenicity of DBE through the formation of reactive half-mustards covalently binding to DNA (Fig. 1.8) (van Bladeren *et al*, 1980, 1981). Mutagenicity towards *Salmonella* is enhanced by the addition of a 100 000g hepatic supernatant fraction (van Bladeren *et al*, 1980) and is considerably decreased in GSH-deficient *Salmonella* strains (Zoetemelk *et al*, 1987). Rat cytosolic components and GSTS catalyze the covalent binding of

DBE to DNA in the presence of GSH with rat isoenzyme 1-2 displaying the highest activity *in vitro* (White *et al*, 1984; Inskeep & Guengerich, 1984; Ozawa & Guengerich, 1983; Wiersma *et al*, 1986; Sundheimer *et al*, 1982).

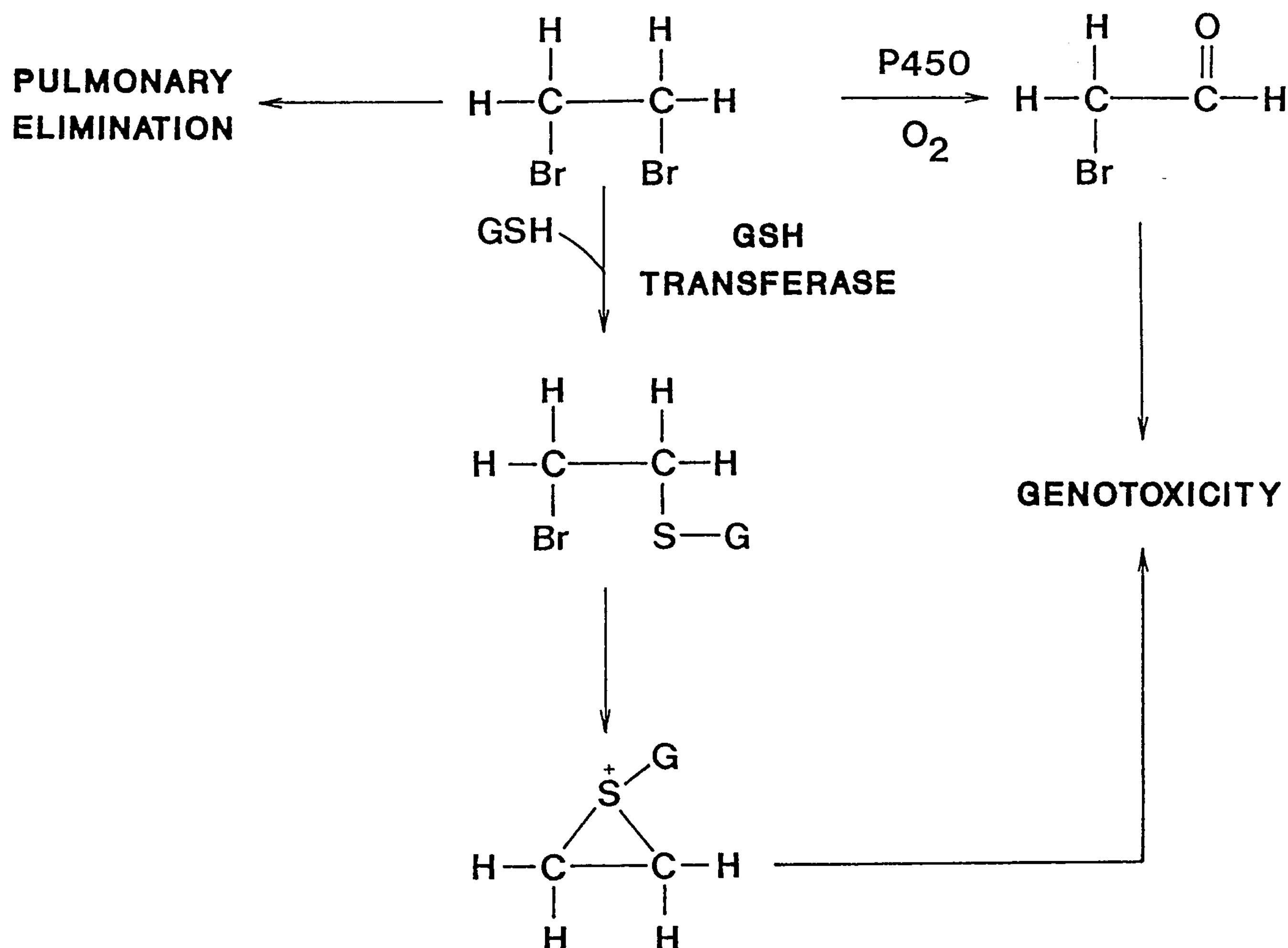


Fig. 1.8: DBE activation and excretion pathways.
(adapted from Pohl & Gillette, 1984)

A single major DNA adduct, identified as S-[2-(N^7-guanyl)ethyl]GSH, was isolated from incubations of DBE, DNA, GSH and GST (Koga *et al*, 1986; Ozawa & Guengerich, 1983). This adduct has been postulated to result from the attack of the DNA guanine N^7 nitrogen on the episulfonium ion formed by the non-enzymatic dehydrohalogenation of the half-mustard produced following the conjugation of DBE with GSH (Koga *et al*, 1986).

The oxidative metabolism of DBE by microsomal proteins also initiates binding of DBE metabolites to cellular RNA, DNA and protein (DiRenzo *et al*, 1982; Hill *et al*, 1978; Wiersma *et al*, 1986). 2-Bromoacetaldehyde and 2-bromoethanol are important intermediates in this process (Banerjee *et al*, 1979; Hill *et al*, 1978).

However, the rate of microsomal DBE metabolism to DNA-binding adducts is much lower than the process mediated by the GSTs (Sundheimer *et al*, 1982; Wiersma *et al*, 1986) and the mixed-function oxidase pathway has been suggested to be the primary route of DBE clearance (Working *et al*, 1986).

1.14 The Characteristics of Bilirubin.

Bilirubin is produced from the catabolism of heme-proteins, of which hemoglobin is the largest component. Bilirubin IXα (Fig. 1.9), formed in mammals by the reduction of biliverdin IXα, is a yellow, toxic compound. This compound exhibits poor solubility in water as a result of internal hydrogen bonding (Fig. 1.9) and requires biotransformation or isomerization before it can be excreted (Brodersen, 1980; Gourley & Odell, 1989; Bonnett *et al*, 1976).

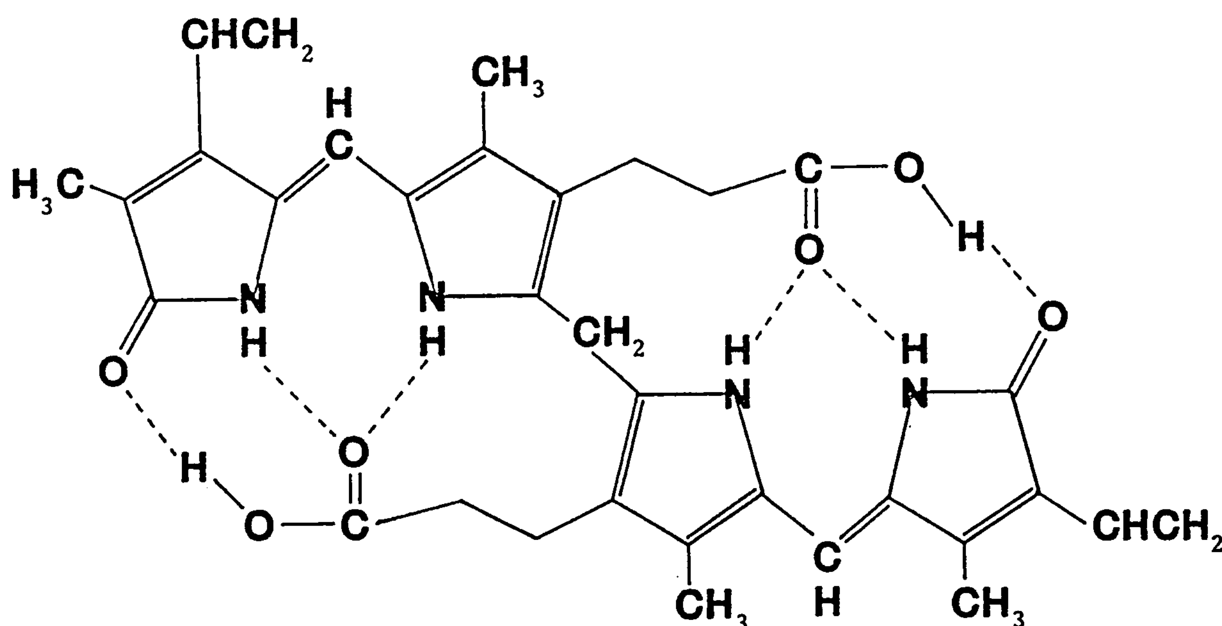


Fig. 1.9: The structure of bilirubin IX-α showing the hydrogen bonding between the relevant atoms.
(adapted from Brodersen, 1980)

Bilirubin is tightly bound to albumin in the blood and conjugated with glucuronic acid in the hepatocytes before excretion in the bile. The hepatic clearance of bilirubin is divided into three distinct, interdependent processes, *viz* the initial uptake of bilirubin into the hepatocytes from the sinusoids, conjugation in the hepatocytes to water-soluble derivatives, and secretion of conjugated bilirubin into the intestinal lumen through the biliary tract (Brodersen, 1980; Gourley & Odell,

1989). The hepatocyte plasma membrane contains receptor-carrier molecules that bind bilirubin and facilitate its transport into hepatocytes (Stremmel *et al*, 1983; Odell & Gourley, 1989) Once in the hepatocytes, bilirubin is bound to the GSTs (Levi *et al*, 1969), one of three distinct groups of bile acid binding proteins which have been identified (Stolz *et al*, 1989).

1.15 **Halothane: Historical Aspects.**

Halothane ($CF_3CHClBr$), an inhalational anesthetic, was introduced into clinical practice in England and the U.S.A. in 1956 and 1958, respectively (Becker & Lamont, 1988). Halothane rapidly achieved general acceptance since it could be administered readily and accurately, was non-explosive, non-flammable, and thought to have minimal side effects (Touloukian & Kaplowitz, 1981). No toxic effects were initially encountered (Raventos, 1956; Stephen *et al*, 1957), but by 1964 over 100 cases implicating halothane in postoperative hepatic injury or hepatitis had been recorded (Blackburn *et al*, 1964; Touloukian & Kaplowitz, 1981). Originally the relationship between halothane and hepatitis was contentious and proponents of halothane thought that unexplained hepatitis following halothane anesthesia was incidental with viral hepatitis or ischemic liver "shock" (Touloukian & Kaplowitz, 1981). However, hepatitis viruses A and B were not be associated with halothane hepatitis* and the lower age distribution and low mortality (less than 1%) of viral hepatis differentiated it from the higher age disposition and elevated mortality (greater than 50% of recognized cases) of halothane hepatitis (Dienstag, 1980).

In the National Halothane Study (1969) an incidence of one fatality per 36 000 halothane administrations within six weeks of anesthesia was detected. However, later studies found an incidence of between one in 6000 and one in 20 000 deaths as a result of severe hepatic dysfunction after halothane anesthesia (Inman & Mushin, 1974; Bottinger *et al*, 1976). Partiality in the National Halothane Study may have decreased the importance of halothane hepatitis (Touloukian & Kaplowitz, 1981; McCaughey, 1972; Becker & Lamont, 1988). Further analysis of the National Halothane Study exposed the increased incidence of hepatitis following multiple halothane anesthesia (McCaughey, 1972). Subsequent studies

---

* Footnote: The term "halothane hepatitis" refers to the form of hepatitis found in some patients after anesthesia with halothane and cannot be explained by other forms of hepatitis. This form of hepatitis has also been referred to as "unexplained hepatitis following halothane" or "halothane-associated hepatitis".

showed that more than 80% of patients with halothane hepatitis had been exposed to halothane more than once (Walton *et al*, 1976; Touloukian & Kaplowitz, 1981). An increased incidence of halothane hepatitis was also found in middle-aged women and the obese (Walton *et al*, 1976; Bottinger *et al*, 1976; Neuberger & Williams, 1984).

Two distinct forms of halothane hepatotoxicity have now been identified: The minor form occurs in approximately 20% of patients after halothane anesthesia with coincident small increases in serum aminotransferase levels (Wright *et al*, 1975; Trowell *et al*, 1975); The more severe form induces hepatic damage and necrosis, *ie* halothane hepatitis (Neuberger & Kenna, 1987).

1.16 **Halothane Metabolism.**

Halothane is metabolized primarily by the hepatic microsomal cytochrome P-450 enzyme system in two pathways, *ie* a reductive and oxidative pathway. Fluoride, 2-chloro-1,1-difluoroethylene (CDF) and 2-chloro-1,1,1-trifluoroethane (CTF) are products of the reductive pathway, whereas trifluoroacetic acid (TFA) and bromide are oxidative metabolites. These compounds are excreted in the urine (TFA, fluoride, bromide) or exhaled in the breath (CDF, CTF). However, metabolic intermediates can also bind to liver macromolecules and ultimately cause hepatotoxicity and necrosis, although the mechanism of toxicity is controversial and both metabolic pathways have been implicated in this process (Fig. 1.10, Fig. 1.11).

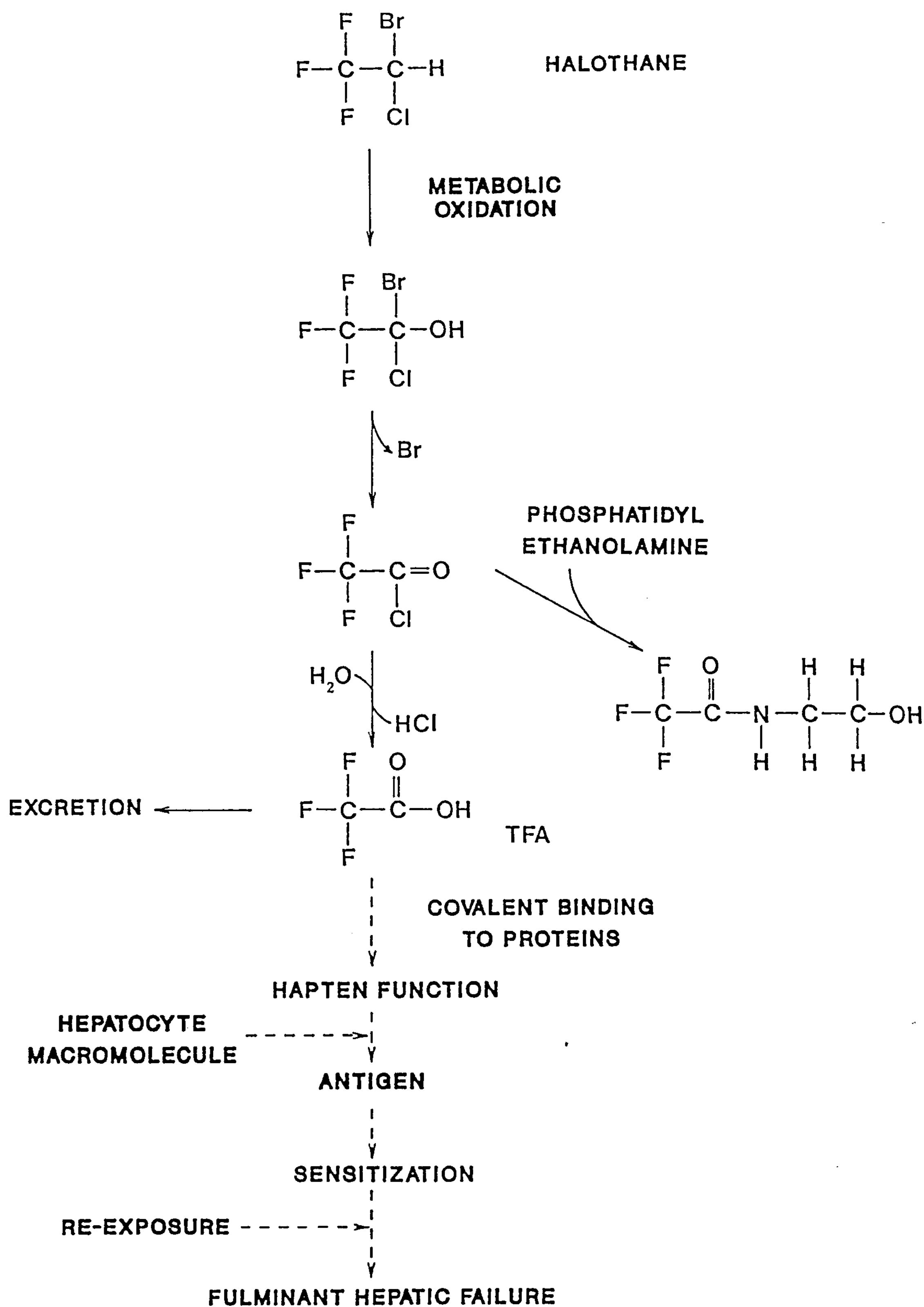


Fig. 1.10: The oxidative metabolism of halothane.
(adapted from Cohen et al, 1975, Owen & Van der Veen, 1986, Börsch & Schmidt, 1987)

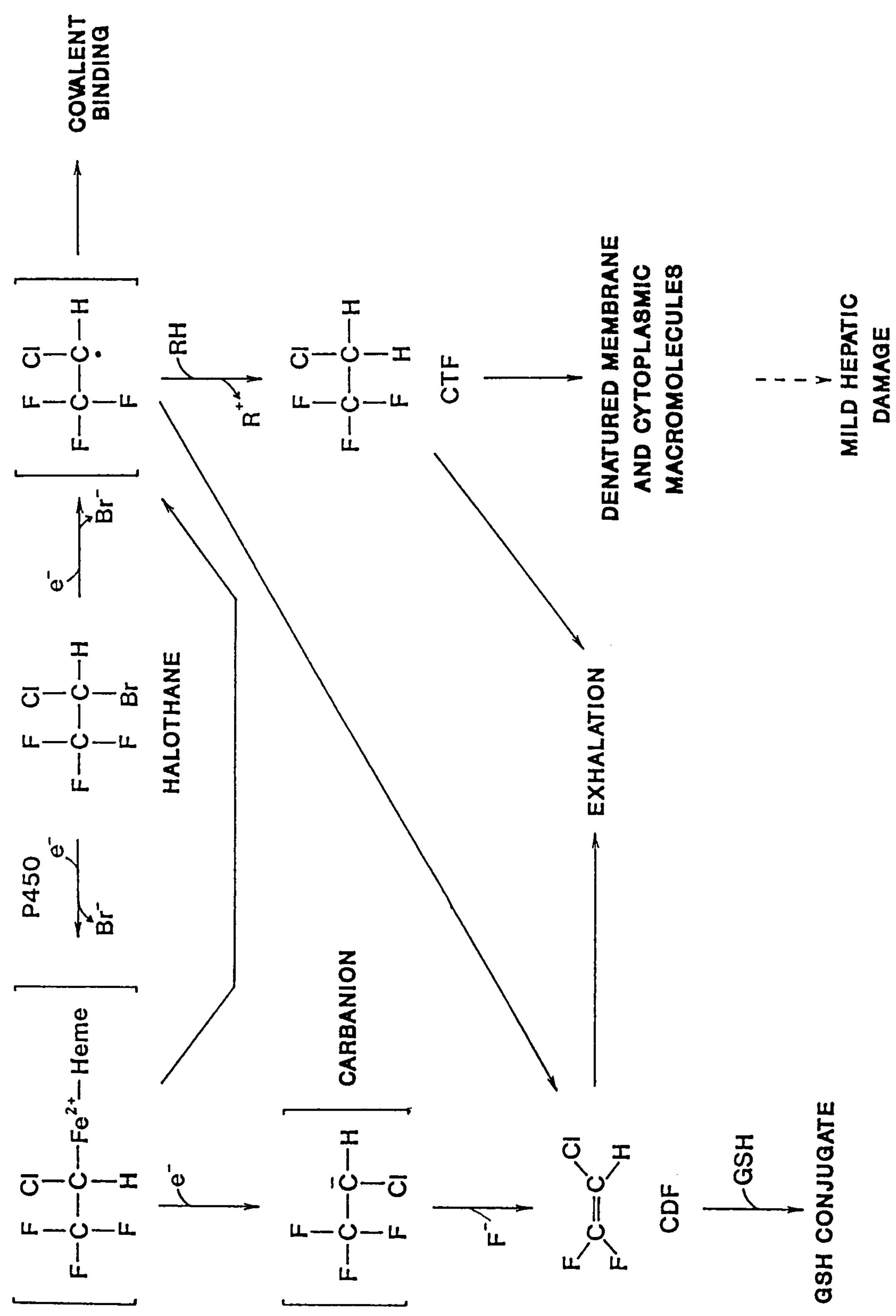


Fig. 1.11: The reductive metabolism of halothane.
(adapted from Cohen et al, 1975, Owen & Van der Veen, 1986, Börsch & Schmidt, 1987)

Reports of increased urinary bromide and TFA levels appeared in the literature a few years after halothane was introduced into clinical practice (Stier *et al*, 1964; Rehder *et al*, 1967). N-trifluoroacetyl-2-aminoethanol and N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine were also identified as halothane metabolites in urine and thought to indicate binding of halothane metabolites to cellular macromolecules such as GSH and phospholipids (Cohen *et al*, 1975). In the presence of oxygen, TFA formation was associated with microsomal cytochrome P-450 levels (Karashima *et al*, 1977). Under anaerobic conditions, halothane adducts covalently bound to microsomal proteins in a cytochrome P-450 dependent process on incubation of microsomes from PB-pretreated rats (Uehleke *et al*, 1973), suggesting halothane metabolism by the hepatic microsomal mixed-function oxidase pathway. The anaerobic dehalogenation of halothane is cytochrome P-450 isoenzyme-specific, as is the formation of the reductive metabolites CTF and CDF (Fujii *et al*, 1981; Ahr *et al*, 1982; Van Dyke *et al*, 1988). Rat hepatic cytochrome P-450 isoenzymes induced by PB or polychlorinated biphenyls (but not 3-methylcholanthrene) catalyzed the reductive defluorination of halothane under low oxygen tensions, while TFA was formed at high oxygen concentrations (Van Dyke & Gandolfi, 1976). Levels of CTF, CDF and lipid peroxidation were increased by halothane anesthesia in PB-induced rats under hypoxic ($O_2 \leq 14\%$) conditions (Gourley *et al*, 1980), although lipid peroxidation has been proposed to result from cell death rather than inducing necrosis (Knights *et al*, 1988; Younes *et al*, 1988). Halothane metabolites bound mainly to microsomal phospholipids after perfusion of rat livers, but protein binding was prevalent after *in vivo* administration of halothane to rats (Van Dyke & Gandolfi, 1974). Incubation of hepatocytes and microsomes with halothane indicated that halothane metabolites bound to both cellular lipids and proteins (McLain *et al*, 1979; Baker & Bates, 1988; Smith *et al*, 1988).

The administration of halothane to PB-induced rats under hypoxic conditions ($O_2 \leq 14\%$) initiated hepatic damage and liver necrosis in male, but not female, rats (Stenger & Johnson, 1972; McLain *et al*, 1979; Ross *et al*, 1979; Jee *et al*, 1980; Ross *et al*, 1984; Hatano *et al*, 1985). Under these conditions halothane hepatotoxicity was associated with hepatic cytochrome P-450 levels and halothane concentration (Ross *et al*, 1984; Jee *et al*, 1980). In the male rat model of halothane hepatotoxicity both PB-induction and hypoxia were required for toxicity (Jee *et al*, 1980; McLain *et al*, 1979; Ross *et al*, 1979).

Under mildly hypoxic conditions the administration of deuterated halothane to PB-induced rats did not alter levels of reductive metabolites (fluoride, CDF, CTF) or affect the degree of hepatotoxicity, while substantially decreasing levels of oxidative metabolites (TFA, bromide). It was therefore proposed that oxidative metabolism of halothane involved the insertion of an oxygen atom at the C-H bond, this is prevented by the stability of the C-D bond. In contrast, reductive metabolism does not involve splitting of the C-H bond, but is implicated in hepatotoxicity (Sipes *et al*, 1980).

In guinea pigs the administration of deuterated halothane reduced plasma TFA and bromide levels and hepatotoxicity, associating oxidative metabolism with halothane hepatotoxicity in the guinea pig (Lind *et al*, 1989). Other studies with rats and guinea pigs have shown that the administration of halothane under normoxic conditions (O_2 = 21%) caused hepatic damage subsequent to oxidative metabolism (Rice *et al*, 1987; Lind *et al*, 1989). Studies with monolayers of rat hepatocytes disclosed that hypoxia, PB-induction and halothane exposure were important factors in cell death (Schieble *et al*, 1988).

1.17 **Halothane Hepatotoxicity.**

The mechanism of halothane hepatitis remains controversial and has been proposed to be the result of either a direct toxicity or a hypersensitive response. Direct toxicity was implicated by the presence of centrilobular necrosis, the short intervals required between exposures, 10% - 20% occurrence of anicteric hepatitis with multiple exposures, and the presence of necrosis in animal models. Hypersensitivity was indicated by the low incidence of halothane hepatitis, the requirement of multiple exposures or rechallenges, the presence of autoantibodies, rash or eosinophilia in some patients, and the presence of antibodies to the plasma membranes of halothane-exposed hepatocytes (Touloukian & Kaplowitz, 1981).

Recent findings have indicated that the fulminant form of halothane hepatotoxicity may have an immunological explanation (Satoh *et al*, 1986) since patients with this form of hepatitis displayed increased occurrence of a liver kidney microsomal and thyroid antibodies and autoimmune complement fixation (Walton *et al*, 1976). Sensitization to halothane-modified hepatocyte constituents was also found in some patients with halothane hepatitis, with antibodies apparently specific for this form of hepatitis and not complementary to liver damage (Mieli-Vergani *et al*,

1980; Vergani *et al*, 1978; Kenna *et al*, 1985). However, patients with halothane hepatitis differed in their patterns of antigen recognition, which was presumed to reflect a idiosyncratic variability in drug metabolism and/or immune response (Kenna *et al*, 1985). Four distinct polypeptides from halothane-treated rabbits were labelled by antibodies in sera from 11 of 14 patients with halothane hepatitis (Kenna *et al*, 1985), while a rat cytochrome P-450 isoenzyme found in the microsomal and plasma membranes was labelled by an antibody to TFA adducts (Satoh *et al*, 1985; Satoh *et al*, 1986). In guinea pigs multiple halothane anesthesia induced the formation of an antibody recognizing a trifluoroacetylated guinea pig serum albumin (Siadat-Pajouh *et al*, 1987).
The immunological determinants of halothane hepatotoxicity have been proposed to include unidentified bound reductive metabolites of halothane or altered autoantigens, the immunological explanation of halothane hepatotoxicity appears to be complicated (Satoh *et al*, 1986).

1.18 **Additional Effects of Halothane.**

Cytochrome P-450 activity is decreased by halothane, possibly as a consequence of the involvement of this group of enzymes in the metabolism of halothane (Ross *et al*, 1979; Stenger & Johnson, 1972; McLain *et al*, 1979). The reaction of halothane with reduced microsomal cytochrome P-450 shows a unusual difference spectrum effected by a trifluoromethyl carbene complex with the reduced hemoprotein. The partial destruction of cytochrome P-450 implicated a highly reactive carbenoid species probably formed by a two-electron reduction of halothane under anaerobic conditions (Mansuy *et al*, 1974; Nastainczyk *et al*, 1978; Loew & Goldblum, 1980). The spectral intermediate was assigned to a ferric cytochrome P-450 and CF_3CHCl^- carbanion complex (Mönig *et al*, 1983; Ruf *et al*, 1984).

Halothane and its metabolites are not mutagenic or carcinogenic (Baden *et al*, 1976; Edmunds *et al*, 1979; Sachdev *et al*, 1980). However, halothane does inhibit lipogenesis (Mapes, 1977), glycolysis (Biebuyck *et al*, 1972) and drug metabolism (Reilly *et al*, 1985), while activating the adenylate cyclase (Rosenberg & Pohl, 1975; Bernstein *et al*, 1985) and protein kinase *c* enzymes (Tsuchiya *et al*, 1988). Halothane anesthesia caused mild hepatic damage in humans (Johnstone *et al*, 1976) and rats (Giler *et al*, 1976), and increased serum GST levels (Allan *et al*, 1987). Multiple, but not single, exposures of halothane to rats has been found to

increase BSP retention in liver perfusates one to three weeks after exposure to halothane (Biebuyck *et al*, 1970).

1.19 The Microperoxidases as Models of Heme Protein Function.

Most organisms require transducing electron-transport chains for adenosine triphosphate production (Salemme, 1977). The proteins comprising such chains utilize iron porphyrins to mediate the electron transport reaction and are part of a group of proteins, the heme-proteins, which catalyze a variety of reactions including oxygen transport (hemoglobin, myoglobin), H_2O_2 metabolism (peroxidases, catalases), oxygen activation and regio-specific insertion (cytochromes P-450), reduction of dioxygen to water (terminal oxidases) and electron transport (cytochrome b_5, cytochromes *c*) (Adams, 1990).

The most intensely investigated electron transporting heme proteins are the highly conserved cytochromes *c* (Salemme, 1977). The greatest homology between the cytochromes *c* from different species is found in the surface structure and charge topography on the side of the protein facing the heme crevice. The mechanism of oxidoreduction has been proposed to involve the direct electron addition to, and withdrawal from, the heme moiety (Salemme *et al*, 1973; Salemme, 1977). The reaction diversity of different heme proteins implies that the apo-protein controls reactivity of the heme by varying the number, nature and strength of axial ligands of the heme or by the diversity of the apo-protein amino acids surrounding the heme moiety (Adams, 1990).

Hemin (ferriprotoporphyrin IX) and related porphyrins were considered as the most relevant model systems for the peroxidases in early studies (Dunford & Stillman, 1976). However, autoxidation and aggregation of these compounds in aqueous solution, the latter requiring detergents or organic solvents for prevention, has required the development of simple hemoprotein analogues (Shack & Clark, 1947; Simplicio & Schwenzer, 1973; Davies, 1973; Adams, 1990). For this purpose specifically designed iron porphyrins or heme peptides, the so-called microperoxidases, have been used (Adams, 1990).

1.20 Properties of the Microperoxidases.

The microperoxidases* are a group of heme peptides prepared by the proteolytic digestion of cytochrome *c* (Adams, 1990). The peptide fragment confers aqueous phase solubility on the heme group and the amino acid composition and chain length can be varied by the use of several proteolytic enzymes and cytochrome *c* from diverse species. The heme moiety is linked to cysteine amino acids by covalent thioether bonds and the Fe^{III+} of the ferriheme-*c* is axially ligated to the imidazole of a histidine (His-18#) (Baldwin *et al*, 1987). Although these peptides were first isolated some 40 years ago (Tsou, 1951), interest in them as models for heme protein function has only emerged in the last decade (Adams, 1990).

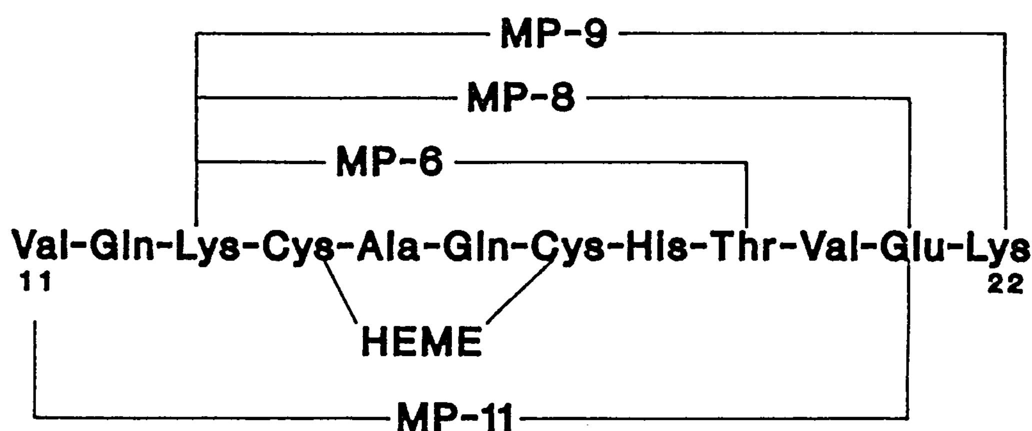


Fig. 1.12: The amino acid sequence of the various microperoxidases obtained by the enzymatic digestion of cytochrome c. The amino acids are numbered according to their positions in the cytochrome c primary sequence. (adapted from Baba et al, 1969)

The microperoxidases dimerize in aqueous solutions, the K_D for MP-8 at pH 7.0 (25 °C) being 1.17×10^5 M^{-1}. However, the concentration of monomers is enhanced with increasing hydrophobicity of the solution (Aron *et al*, 1986; Baldwin *et al*, 1987). Dimerization of MP-8 takes place by the stacking or oblique orientation of the π-π system of the tetrapyrrole ring (Urry & Pettegrew, 1967; Adams, 1990). MP-9 and MP-11 aggregate by the intermolecular coordination of amino groups to the iron atom in a nearly head-to-tail alignment of the heme planes (Urry, 1967; Peterson *et al*, 1980, 1983; Wilson *et al*, 1977). At concentrations required for catalytic studies ($< 10^{-6}$ M) MP-8 is water-soluble and

* Footnote: The microperoxidases will be referred to by the abbreviated format MP, *ie* MP-8 refers to microperoxidase-8. The numeral in the nomenclature refers to the number of amino acids present in the peptide.

Footnote: The amino acids of the microperoxidases are generally numbered according to their position in cytochrome *c*.

essentially monomeric in aqueous and aqueous/methanol solutions (Aron *et al*, 1986). MP-8 has been used as a model for the cytochromes (Harbury & Loach, 1960), hemoprotein anion-binding (Blumenthal & Kassner, 1980) and the peroxidase enzymes (Baldwin *et al*, 1985).

Three reversible, concentration-independent pK values have been determined for MP-8 and attributed to the deprotonation of His-18 (and its coordination to Fe^{III}), bound H_2O, and bound His-18 to form a imidazolate complex (Baldwin *et al*, 1986). The coordination sphere of Fe^{III} consists of His-18 and H_2O as axial ligands in MP-8 and MP-9 (Aron *et al*, 1986; Baldwin *et al*, 1987). MP-11 also undergoes at least three pH-dependent transitions ascribed to the binding of a deprotonated imidazole group to the ferric iron and the binding of the two available amino groups (α-NH_2 of valine and ϵ-NH_2 of lysine) (Wilson *et al*, 1977).

1.21 **Microperoxidase Purification.**

The microperoxidases are prepared by the digestion of cytochrome *c* with a range of proteolytic enzymes. MP-11 and MP-9 are prepared by pepsin and trypsin digestion of cytochrome *c*, respectively. MP-8 is obtained by the tryptic digestion of MP-11, and MP-6 by nagarse (a non-specific protease) digestion of either MP-8 or cytochrome *c*. Most methods require long incubation times ($\geq$ 12 hours), although a trypsin-Sepharose column has been used requiring approximately 5 hours. Several purification procedures, including protein precipitation, molecular sieving, ion-exchange chromatography and dialysis, are usually required, most methods appearing to require at least two days to purify the microperoxidases (Baba *et al*, 1969; Plattner *et al*, 1977; Peterson *et al*, 1980, 1983). However, the use of reverse-phase HPLC as a purification step has substantially decreased the preparation time for MP-8 from cytochrome *c* ($\leq$ 30 h), at the same time increasing yield (> 98%) and purity (> 90%) of the sample (Adams *et al*, 1988).

1.22 Motivation for Study.

In this thesis a number of topics relating to the GSH S-transferases were covered. The reasons for this diversity were two-fold. In the first instance our laboratory started working on the GSTs one year before my arrival and therefore were still in the process of setting up the laboratory with respect to work related to the GSTs. Secondly, my initial supervisor, Associate Professor K.M. Ivanetich, emigrated from South Africa during 1988. It was therefore necessary to find a second, resident supervisor, Dr. P.A. Adams. The work with Dr. Adams concentrated on heme protein and peptide chemistry.

Initial studies related to the interactions of the anesthetic halothane with the GSTs. The prime motive for this was a report of halothane decreasing the hepatic uptake of BSP (Biebuyck *et al*, 1970). It was hypothesized by us that this finding possibly reflected a decrease in hepatic GST levels as BSP is known to bind to certain GST subunits (Jakoby *et al*, 1976; Mannervik & Jensson, 1982). In addition, other halocarbons such as carbon tetrachloride (Younes *et al*, 1980) and 1,2-dibromoethane (Ivanetich *et al*, 1984; Botti *et al*, 1982) had been demonstrated to inactivate GSTs in an isoenzyme-dependent manner. (Chapter 3)

Incubations of GSTs with halothane (this study) and 1,2-dibromoethane (Ivanetich *et al*, 1984) appeared to have similar effects on rat hepatic GST activity. Therefore, following on the studies of the reversible effects of halothane on GST activity, the reversible effects of DBE were also analyzed. (Chapter 4)

The binding of bilirubin to the GSTs and the resultant inhibition of GST activity has been widely reported (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982, 1983; Ketley *et al*, 1975; Arias *et al*, 1976). However, GST inhibition by bilirubin was prevented by the addition of proteins to incubations and it was proposed that a form of "enzyme memory" resulted in the observed protective effect (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982, 1983). Our analysis of the relevant data appeared to indicate that the results had possibly been incorrectly analyzed and that a simpler explanation could explain the observed phenomenon. Thus our investigation of bilirubin inhibition and protein protection. (Chapter 5)

The kinetic mechanism of only a few GSTs have been elucidated (Mannervik, 1985; Boyer & Kenney, 1985). Since GSTs ρ and π had been proposed to be similar or identical (Guthenberg & Mannervik, 1981) and the kinetic mechanism

of isoenzyme π was in the process of being elucidated in our laboratory (Ivanetich & Goold, 1989), the mechanism of GST ρ was investigated. The kinetic mechanism of GST π and GST ρ was found to be virtually identical. (Chapter 6)

The heme peptides (microperoxidases) are known to bind to human serum albumin and GST π (Adams *et al*, 1989; Adams & Goold, 1990). An additional discriminatory mechanistic tool, in the form of microperoxidase binding and inhibition, was therefore used to investigate the similarity of these two isoenzymes at the structural level and to further investigate mechanisms and binding of non-substrate ligands to the GSTs. (Chapter 7)

Materials and Methods.

Materials.

Chemicals.

Isoflurane, enflurane, methoxyflurane: Abbott Laboratories, Kent, U.K.; *trans*-4-phenyl-3-buten-2-one: Aldrich Chemicals, Gillingham, Dorset, U.K.; Diaflo ultrafiltration membranes: Amicon Corporation, Lexington, Mass., USA; Ammonium persulfate, DTT, DBE (1,2-dibromoethane), *bis*-acrylamide, β-mercaptoethanol: BDH Chemicals, Poole, England; Acetonitrile; Beckman; Bio-rad protein determination kit (including γ-globulin protein standard), Bio-rad silver staining kit: Bio-rad Laboratories, München, West Germany; DCNB: EGA-CHEMIE, Steinheim/Albuch, West Germany; Cumene hydroperoxide (70% in cumene): Fluka AG, Buchs, Switzerland; Halothane: Maybaker, South Africa; CDNB, hydrogen iodide, DTNB, SDS, acrylamide, TFA, TEMED, DMSO, Folin-Ciocalteu's phenol reagent: Merck, Darmstadt, West Germany; BSA: Miles Research Products, Cape Town, South Africa; 1-Chloro-2,2-difluoroethylene: PCR Research Chemicals, Gainsville, Florida, USA; Sephadex G-25, PBE 118 & PBE 94 chromatofocusing resins, Pharmalyte pH 10-8.5 chromatofocusing buffer, Polybuffer 96, Polybuffer 74, epoxy-activated Sepharose 6B: Pharmacia Fine Chemicals, Uppsala, Sweden; Acetonitrile: Rathburn Chemicals Ltd., Walkerburn, Scotland; Blood transfusion bags: SABAX, Johannesburg, RSA; GSH, GSH Sepharose, BSP GSH Sepharose, trypsin, pepsin, cytochrome *c*, HSA, TEAE cellulose ion-exchange chromatography resin, DTE, bilirubin, human hemoglobin, bovine pancreatic ribonuclease A, aldolase, DTE, GSH reductase, MP-11, GSH reductase, NADPH, dialysis tubing: Sigma Chemical Company, St. Louis, MO, USA; DEAE ion-exchange chromatography resin (DE 52), CM-cellulose ion-exchange chromatography resin (CM 52): Whatman, Maidstone, Kent, U.K.:

HPLC Columns:

Synchropak AX-300 HPLC column: 25 cm x 4.6 mm, weak anion exchange column; Synchrom Incorporated, Linden, IN, USA.

Microbondpak C_{18} HPLC column : 10 cm x 8.0 mm, reverse phase column; Waters Associates, Milford, MA, USA.

Methods.

All reagents used were analytical reagent (AR) grade, aqueous solutions were made up using de-ionized, pyrogen-free water, unless otherwise stated. Concentrations given as percentages indicate either weight/volume or volume/volume.

2.1 Assays for GST Activity.

2.1.1 Standard Assay Conditions.

All spectrophotometric assays were performed at 25 °C in 0.1 M potassium phosphate buffer of the indicated pH (Table 2.1). Substrates with limited water solubility were prepared weekly as stock solutions in ethanol (3.33% ethanol final concentration in assay medium) and protected from light. GSH solutions were made fresh daily in degassed water or phosphate buffer and neutralized with KOH. The electrophilic substrates were equilibrated with buffer (in cuvettes), and GSH was added immediately before assays were initiated by the addition of enzyme. Absorbance measurements were routinely performed using a Beckman UV 5230 or Unicam SP1800 spectrophotometer (Habig & Jakoby, 1981; Askelöf *et al*, 1975). The electrophilic substrates were made up in ethanol and added to phosphate buffer at 25 °C. GSH (150 μl) was added prior to the addition of diluted enzyme (50 μl) used to start the assay.
For kinetic studies, experiments were performed on a centrifugal analyzer (see Section 2.1.3).

The CDNB, DCNB and *trans*-4-phenyl-3-buten-2-one assays have been reported as apparently linear for at least three min at absorbance changes of less than 0.05 per min (Habig *et al*, 1974). The CDNB assay was linear in our system for only up to one min. Enzymic rates were generally measured over one min. Reactions in the absence of enzyme were significant and linear for at least five min under the conditions used.

2.1.2 Cumene Hydroperoxide Assay.

The cumene hydroperoxide assay differs from the above in that it is a coupled GSH peroxidase in which NADPH oxidation is measured as a function of peroxidase activity.
The enzyme was preincubated in a cuvette with 100 µl EDTA (3.0 mM), GSH (1.0 mM), NADPH (0.11 mM) and GSH reductase (13 ng protein/100 µl) and buffer (100 µl) at room temperature for 10 min prior to starting the assay by addition of cumene hydroperoxide (0.1 mM in 1% EtOH) (Wendel, 1981). All solutions were made up fresh daily. The reaction was linear for one min at absorbance changes of less than 0.05 absorbance units per min (own observation).

Table 2.1: Conditions for spectrophotometric GST assays.

	Substrate			
Conditions	CDNB	DCNB	trans-4-phenyl-3-buten-2-one	Cumene hydroperoxide[a]
[Substrate] (mM)	1.0	1.0	0.05	-
[GSH] (mM)	1.0	5.0	0.25	1.0
Wavelength (nm)	340	345	290	340
Extinction coefficient ($mM^{-1}.cm^{-1}$)	9.6	8.5	-24.8	-6.0
Phosphate buffer (pH)	6.5	7.5	6.5	7.0[b]

[a] A further description of the cumene hydroperoxide assay is given below.
[b] The phosphate buffer used in the cumene hydroperoxide assay also contains 0.1 M KCl.
(adapted from Habig *et al*, 1974, Wendel, 1981)

2.1.3 **Centrifugal Analyzer.**

For detailed kinetic studies of erythrocyte GST ρ, a MultistatR Plus centrifugal analyzer was used (Instrumentation Laboratory, Lexington, MA, USA). The Multistat^{R} Plus System is an automated centrifugal absorbance detector used to measure simultaneously absorbance changes of multiple samples. Reactants were loaded into disposable plastic rotors; These contain 20 separate cuvettes, divided by a slight ridge. This division allows for separation of reagents to be maintained until the start of the assay. After loading the cuvettes were placed in the instrument and the rotor allowed to temperature equilibrate (30 °C). Reaction was initiated automatically in the instrument using a rapid spin/stop procedure. Enzyme activity towards CDNB was measured in triplicate at 340 nm at 30 ± 0.2 °C at 6 second intervals for 1 min. Non-enzymic absorbances were measured at 30 second intervals over 5 min. Absorbance measurements were started 10 seconds after mixing of the reagents.
CDNB (0.2 - 2.0 mM) was dissolved in DMSO (2% v/v in assay; protected from light) and mixed into 0.1 M phosphate buffer (pH 6.5). GSH was dissolved in degassed phosphate buffer and the solution neutralized with KOH. The enzyme was diluted in buffer to the correct concentration before use. Assay volumes were as follows: premixed buffer and CDNB (180 μl), GSH (10 μl) and diluted enzyme (10 μl). All solutions, with the exception of buffer, were made fresh daily.
The CDNB solution was added to the outer chamber of the rotor with the enzyme added to the inner compartment (see diagram of Multistat rotor below). GSH was added to the CDNB solution immediately before loading the rotor into the instrument. The enzyme was kept separate from the other constituents until the samples were mixed by the instrument.
Results were fitted to a straight line by linear regression (using Lotus 1-2-3^{R}). Enzyme rates were corrected for the non-enzymic reaction.

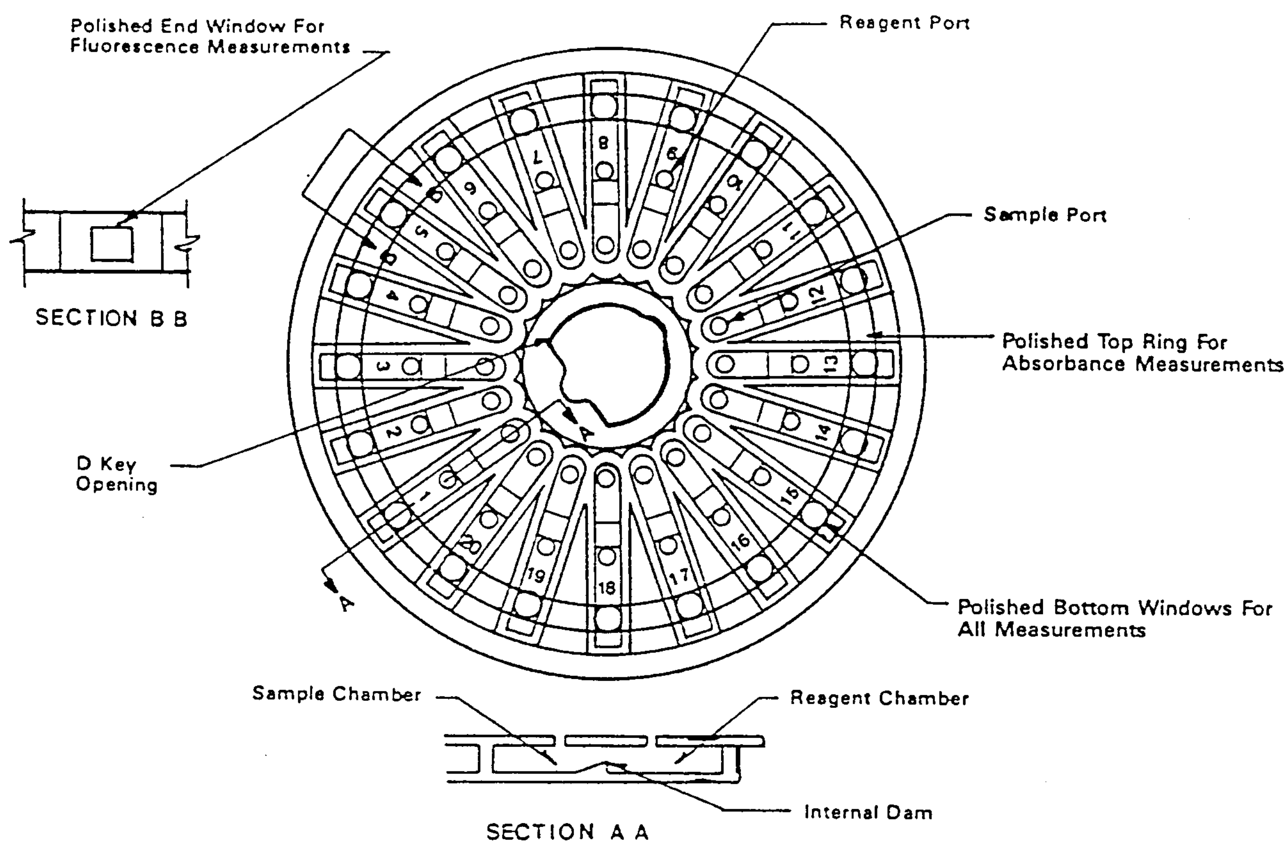


Fig. 2.1: Diagrammatic representation of the Multistat^{R} centrifugal analyzer rotor.
(obtained from the instruction manual)

2.2 Protein Concentration Assays.

2.2.1 Lowry Protein Assay.

The Lowry procedure was used for all protein assays, except for GST ρ studies and unless otherwise stated, were carried out using the Lowry method (Lowry *et al*, 1951; Chaykin, 1966).
A 2.0% sodium carbonate solution in 0.1 M NaOH (solution A) was combined with a 1.0% copper sulfate solution (solution B) and a 2.0% sodium tartrate solution (solution C) in the ratio 50:1:1 (A:B:C) to give solution D, made fresh daily. Solution E consisted of 1 part Folin-Ciocalteu's reagent diluted with 2 parts water.

The diluted protein sample (0.5 ml) was mixed with solution D (5 ml), and 10 min later solution E (0.5 ml) was added with mixing. The absorbance of the sample was measured after 10 min at 600 nm and the data corrected for the relevant control, which did not contain protein.
BSA (in water) was used as a protein standard (0 - 100 μg/ml). The concentration of BSA standards was measured at 280 nm (1 mg/ml = 0.667 absorbance units).

2.2.2 **Bio-rad Protein Assay.**

The Bio-rad protein assay was performed according to the suppliers instructions. In the standard assay (20 - 140 μg protein, 200 - 1400 mg/ml) samples (0.1 ml) were mixed with 5.0 ml dye reagent (5-fold dilution). In the microassay (1 - 20 μg protein; <25 μg/ml) samples (0.8 ml)were mixed with 0.2 ml concentrated dye reagent (The absorbance measured between 5 and 60 min later at 595 nm).
Since the dye adsorbs on glass and plastic, absorbances were measured in a cuvette pre-equilibrated with the dye concentrate.

2.2.3 **Spectrophotometric Assays.**

The following equation was used to estimate protein concentrations in samples prior to quantification by the above methods:

$$[\text{protein}]\ (\text{mg/ml}) = 1.45 \times A_{280\ \text{nm}} - 0.74 \times A_{260\ \text{nm}}$$

(Thorne, 1978)

2.3 Protein Gel Electrophoresis.

2.3.1 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE gels were generally run using a discontinuous buffer system in a modification of the method by Laemmli (1970), as described by Hames (1981), with the stacking and separating gels, containing 5% and 12.5% acrylamide, respectively, prepared from a stock solution containing 30% acrylamide, 0.8% *bis*-acrylamide and 0.1% SDS. The stacking gel buffer consisted of 125 mM Tris.HCl and 0.1% SDS, pH 6.8, and the separating gel buffer 375 mM Tris.HCl, 0.1% SDS and 20% glycerol (pH 8.8). The electrode buffer contained 25 mM Tris.HCl (pH 8.3), 192 mM glycine and 0.1% SDS.
In order to facilitate polymerization the acrylamide solutions were mixed with ammonium persulfate (0.1% stock solution, final concentration between 0.0005% and 0.001%) and degassed on an aspirator for one min. Solutions were filtered through 0.2 μm filters before TEMED (10 μl) was added and the gels poured immediately. The resolving gel was usually covered with a 0.1% SDS solution and allowed to polymerize overnight. The stacking gel was prepared in the same way and poured onto the polymerized separating gel. Samples were loaded onto the stacking gel, electrophoresis buffer was added to the system and electrophoresis started.
Protein samples (approximately 10 μg per protein band in less than 25 μl) were diluted 2-fold in 125 mM Tris.HCl buffer containing 4% SDS and 24% glycerol (pH 6.8). β-Mercaptoethanol (5%), used as a reductant, and bromophenol blue, a marker dye, were added to samples before boiling for 3 min and loading. Samples with low protein concentrations were lyophilized and redissolved in approximately 20 μl water and treated in the same way.
Initially a constant current of 25 mA was applied to the gels; This was increased to 30 mA when the protein entered the resolving gel. Samples were electrophoresed until the bromophenol blue reached the lower border of the gel. Subsequently gels were stained for protein detection as described below.

2.3.2 Protein Staining.

2.3.2.1 Coomassie Blue Stain.

Polyacrylamide gels were stained with a solution of 50% methanol, 20% acetic acid and 0.12% Coomassie Blue in water for at least 2 hours before non-specific stain was removed with a solution of 10% methanol and 10% acetic acid and the gel stored in water (Hames, 1981).

2.3.2.2 Bio-rad Silver Stain.

In samples containing too little protein for detection by the Coomassie Blue stain the more sensitive Bio-rad silver stain method was used.
The Bio-rad silver stain (Bio-rad bulletin 1089) was used in kit form containing the following solutions: Fix I (40% methanol and 10% acetic acid in water); Fix II (10% ethanol and 5% acetic acid in water); Oxidizer (this solution was supplied as a 10-fold concentrate, composition unknown); Silver reagent (supplied as a 10-fold concentrate, composition unknown); Developer (supplied in powder form and dissolved in water); Stopping solution (5% acetic acid in water).
The gels were soaked in fix I for 30 min, followed by fix II (twice for 15 min), which could be left with the gel overnight. Subsequently the gel was gently shaken in oxidizer for 5 min, followed by silver solution for 20 min and developer (twice for 5 min). The second treatment with developer was continued until protein bands became visible and the reaction was then stopped by addition of the stopping solution (15 min). Gels were stored in water.

2.4 Glutathione Assays.

Two methods were used to measure GSH in the millimolar and micromolar concentrations ranges. The first method (see below) was used to gauge the effect of dialysis and chromatography on GSH levels. Halothane metabolism, *ie* conjugation with GSH, was determined by the second method.

2.4.1 Glutathione Concentrations in the Millimolar Range.

Protein was removed from GSH samples (1.5 ml) by precipitation with 0.1 ml TCA (33%) and centrifugation at 2000 rpm for 5 min. A 0.1 ml aliquot of the supernatant was mixed with 1.5 ml of a 0.05 mM DTNB solution in 0.1 M phosphate buffer, pH 6.5, and the absorbance measured at 412 nm after 30 min (Ellman, 1959; van Bladeren *et al*, 1980).

2.4.2 Glutathione Concentrations in the Micromolar Range.

Lyophilized samples were dissolved in 10 mM HCl to a GSH concentration of 0.3 to 1.5 μM and aliquots (300 μl) added to test tubes at 25 °C. Reagents 1 and 2 (300 μl each) (see below) were added to the sample simultaneously and the absorbance at 412 nm measured after 15 min.
Reagent 1 contained sodium phosphate buffer (110 mM Na_2HPO_4/40 mM NaH_2PO_4) at pH 7.2 with 15 mM EDTA, 0.04% BSA and 0.3 mM DTNB (Ellman's reagent), whereas reagent 2 comprised 15 mM imidazole.HCl, 1 mM EDTA, 0.02% BSA, GSSG reductase (1.2 IU/ml) and 0.6 mM NADPH (pH 7.2). NADPH and GSH reductase were added to reagent 2 daily (Tietze, 1969; Brehe & Burch, 1976).
Incubation mixtures containing GSH were diluted with 10 mM HCl and an aliquot lyophilized. The samples were redissolved in 10 mM HCl and the GSH concentration determined as described.

2.5 Anesthesia of Rats with Halothane.

Male Long-Evans rats (200 ± 10 grams) were starved for 24 hours before being anesthetized with halothane (1% in medical air, 1.25 MAC) in standard anesthetic tanks. Halothane/air was delivered at a flow rate of 6 l/min under controlled conditions of temperature and humidity.

2.6 Purification of Rat Hepatic GST Isoenzymes.

2.6.1 Cytosolic Preparation.

Rat hepatic cytosol was prepared from livers of male Long-Evans rats (200 ± 10 grams). The livers were washed with 10 mM Tris.HCl buffer, pH 7.8, homogenized in the same buffer (20% homogenate) and centrifuged at 18 000g (Beckman J2-21 centrifuge; JA-20 rotor) for 15 min. The supernatant was centrifuged at 100 000g (Beckman L8-70M ultracentrifuge; Type 65 rotor) for 1 hour to remove the microsomes. The supernatant from the 100 000g centrifugation was used as cytosol for further experiments or purification of the transferase isoenzymes (Ernster *et al*, 1962).

2.6.2 Purification of the Mixture of Rat GST Isoenzymes*.

2.6.2.1 Method 1.

Approximately 20 ml cytosol was applied to a Sephadex G-25 column (1.6 cm x 100 cm) equilibrated with 10 mM Tris.HCl, pH 7.8, to remove low molecular weight contaminants. Protein eluting in the void volume was collected and loaded onto a S-hexyl GSH affinity column equilibrated with 10 mM Tris.HCl, pH 7.8, and washed (usually overnight) with 10 mM Tris.HCl containing 0.2 M NaCl, pH 7.8, to remove non-specifically bound proteins. The GSH S-transferases were eluted with the Tris/NaCl buffer containing 5 mM S-hexyl GSH (pH 7.8). The eluted enzyme was chromatographed on a second Sephadex G-25 column to remove S-hexyl GSH, which is an inhibitor of the transferases. The latter column was equilibrated with 10 mM Tris.HCl buffer, pH 7.8, for subsequent storage of the GST mixture, or equilibrated with the relevant buffers for further purification by chromatofocusing (Guthenberg & Mannervik, 1979; Jensson *et al*, 1982).

---

* The family of rat GST's which have been partially purified by affinity chromatography are referred to as the GST mixture. The purified isoenzymes are referred to as GST isoenzymes, while cytosolic GST's are the GST enzymes present in cytosol.

2.6.2.2 **Method 2.**

Cytosol was prepared in 10 mM Tris.HCl, pH 8.0, and filtered through cheese-cloth to remove free floating lipids. The cytosol was applied to a DEAE-cellulose column (5 cm x 20 cm) equilibrated with 10 mM Tris.HCl, pH 8.0, and the void volume collected (Habig *et al*, 1974).
The collected protein was concentrated on an Amicon PM-10 ultrafiltration membrane and dialyzed against 25 mM sodium phosphate buffer, pH 9.4, before purification of the GST mixture on a GSH affinity column (1 cm x 13 cm) equilibrated with the same buffer. The affinity column was washed overnight with 25 mM sodium phosphate containing 150 mM NaCl, pH 9.4, and the enzyme eluted with the latter buffer containing 100 mM GSH. Before storage the enzyme was dialyzed against 10 mM potassium phosphate buffer, pH 6.7, containing 30% glycerol (w/v) and 1 mM EDTA (Simons & Vander Jagt, 1977).

2.6.3 **Preparation of Affinity Matrices.**

2.6.3.2 **Preparation of S-Hexyl GSH.**

GSH was dissolved in water to a concentration of 1.0 M and 4.0 mmoles NaOH added to 2 ml of this solution with stirring at room temperature. Ethanol was added dropwise with stirring to form a cloudy precipitate, following which an equimolar amount of 1-iodohexane was added over 30 min with stirring. The pH of the solution was reduced to 3.5 with 47% hydriodic acid, and the solution left overnight at 4 °C. The resulting precipitate was removed by filtration and washed with water prior to recrystallization from water/ethanol. The crystals were dried and stored in a dessicator at -20 °C (Vince *et al*, 1971).

2.6.3.2 **S-hexyl GSH Sepharose Affinity Matrix.**

The S-hexyl GSH affinity matrix was prepared according to the instructions of the manufacturer. S-hexyl GSH (0.011 mM) was dissolved in 0.1 M sodium carbonate.NaOH, pH 10.6, and added to washed epoxy-activated Sepharose 6B. Thirty ml of ligand was added to 15 g Sepharose. The reaction was left to proceed at 30 °C for 30 hours with gentle shaking. The resin was washed with approximately 400 ml each of 0.1 M sodium carbonate (pH 10.6), 0.1 M sodium

borate containing 0.5 M NaCl (pH 8.0), 0.1 M sodium acetate containing 0.5 M NaCl (pH 4.0), and water.

2.6.3.3 GSH-Sepharose Affinity Matrix.

Epoxy-activated Sepharose 6B (4 g) was washed on a Buchner funnel with 500 ml water and 40 ml 44 mM phosphate buffer (pH 7.0). The gel was transferred to a 50 ml flask and the volume adjusted to 20 ml with the latter buffer, and nitrogen bubbled through the suspension for 5 min. A 4 ml solution of GSH (400 mg/4 ml), pH 7.0, was added to the gel suspension and coupled for 24 hours at 37 °C with shaking. The coupled gel was washed with 100 ml water and the active groups blocked by treatment with 1.0 M ethanolamine (4 hours) before washing (100 ml each) with 0.1 M sodium acetate/0.5 M KCl (pH 4.0) and 0.1 M sodium borate/0.5 M KCl (pH 8.0), with storage at 4 °C in water (Simons & Vander Jagt, 1977).

2.6.4 Separation of the Rat GST Isoenzymes by Chromatofocusing.

The GST isoenzymes were separated on chromatofocusing resin PBE 118 (1 cm x 30 cm) with a pH 11 - 8 gradient, according to the specifications of the supplier. The resin was equilibrated with 25 mM triethylamine.HCl (pH 11.0). The proteins were eluted with Pharmalyte pH 10 - 8.5 buffer which had been adjusted to pH 8.0 and diluted 80-fold with water (Jensson *et al*, 1982; Mannervik & Jensson, 1982).

Alternatively, PBE 94 resin (1 cm x 30 cm) was equilibrated with 25 mM ethanolamine.HCl, pH 10.0, and the isoenzymes eluted with a 10% solution of Polybuffer 96 and Polybuffer 74 (6:4 v/v, pH 5.5) (Ivanetich *et al*, 1984).

In both chromatofocusing methods, eluate was monitored for protein ($A_{280\ nm}$) and GST activity (CDNB assay).

2.6.5 Separation of GST Isoenzymes by Ion-Exchange Chromatography.

Cytosol was prepared in water and chromatographed on a DEAE-cellulose column (9 cm x 23 cm) equilibrated with 10 mM Tris.HCl (pH 8.0). Proteins eluting in the void volume were collected (this fraction contained approximately 70% of the loaded GST activity) and concentrated on a hollow fibre concentrator. (Although this concentration method resulted in the loss of enzyme activity, the Amicon ultrafiltration system used later in the separation procedure was too slow for the volumes which were concentrated at this initial stage).
The concentrate was dialyzed against 10 mM Tris.HCl, pH 9.4, before chromatography on TEAE-cellulose (2 cm x 23 cm). Isoenzymes with subunits 1 and 2 were eluted in the buffer wash; Isoenzymes comprising subunits 3 and/or 4 were eluted with 40 mM Tris.HCl (pH 8.0).
Both enzyme fractions (*ie* isoenzymes 1-1, 1-2 and 2-2 in one fraction, and isoenzymes 3-3, 3-4 and 4-4 in the other fraction) were loaded onto GSH affinity columns (2 cm x 4.7 cm). The matrix was equilibrated with 25 mM sodium phosphate, pH 9.4, and washed with 25 mM sodium phosphate containing 150 mM NaCl (pH 9.4). Both GST fractions were eluted with the phosphate/NaCl buffer containing 100 mM GSH, pH 9.4, concentrated on Amicon PM-10 ultrafiltration membranes and dialyzed against 10 mM potassium phosphate, pH 6.7 (36 hours).
The isoenzymes were separated on two CM-cellulose columns (2.5 x 36 cm) equilibrated with 10 mM potassium phosphate, pH 6.7, and washed with equilibration buffer (500 ml) before application of a linear 0 - 75 mM KCl gradient in equilibration buffer (1000 ml). Protein peaks (280 nm) were pooled before concentration and dialysis against 10 mM potassium phosphate buffer (pH 6.7) containing 30% (w/v) glycerol and 1 mM EDTA (36 hours).
(Boyer *et al*, 1983; Boyer & Kenney, 1985; Habig *et al*, 1974)

GST isoenzymes purified by chromatofocusing and ion-exchange chromatography were characterized by their elution profiles, subunit size on SDS-PAGE, and substrate specificities for CDNB, DCNB, *trans*-4-phenyl-3-buten-2-one and cumene hydroperoxide (Jensson *et al*, 1982; Mannervik & Jensson, 1982).

2.7 Purification of Human Erythrocyte GST s.

Blood was collected from healthy individuals into 500 ml blood bags containing a dextrose/phosphate solution as the anticoagulant (70 ml per unit of blood). Blood was centrifuged at 500g for 10 min (Beckman J2-21 centrifuge; JA-10 rotor) and plasma and buffy coats removed, followed by filtration through cotton wool to remove leukocytes. The erythrocytes were washed four times with six volumes of 10 mM potassium phosphate containing 140 mM NaCl (pH 7.0) by centrifugation at 500g for 10 min (Beckman J2-21 centrifuge; JA-10 rotor).
The washed erythrocytes were hemolyzed using six volumes of 5 mM potassium phosphate containing 1.4 mM β-mercaptoethanol, pH 7.0, and dialyzed against 22 mM potassium phosphate containing 1.4 mM β-mercaptoethanol (pH 7.0).
The dialyzed hemolyzate was centrifuged at 10 000g for one hour (Beckman J2-21 centrifuge; JA-10 rotor) and the supernatant fraction chromatographed on a GSH affinity column (4 cm x 4 cm). The affinity column was washed overnight with dialysis buffer and the enzyme eluted with 10 mM GSH in 50 mM Tris-KOH (pH 9.6). The enzyme was subsequently dialyzed against 10 mM potassium phosphate buffer, pH 7.4, before chromatography on a BSP-GSH agarose affinity column (4 cm x 4 cm) equilibrated with the same buffer. GST ρ eluted as a sharp peak immediately after the void volume. The column was washed thoroughly with the equilibration buffer and the basic erythrocyte GST eluted with 10 mM potassium phosphate containing 10 mM GSH, pH 7.4 (Clark *et al*, 1977; Awasthi & Singh, 1984). Both enzyme fractions were concentrated on Amicon PM-10 ultrafiltration membranes before dialysis against 5 mM potassium phosphate containing 1.4 mM β-mercaptoethanol, pH 7.0 (Awasthi & Singh, 1984).

An aliquot of GST ρ was dialyzed against 5.5 mM citrate-phosphate buffer containing 1 mM DTT and 5 mM GSH (pH 5.85). A Synchropak AX-300 anion-exchange HPLC column (4.6 mm x 250 mm) was equilibrated with the same buffer (at least 6 hours) and an aliquot of the enzyme (100 - 200 μg protein) loaded onto the column. The column was washed for 5 min with the equilibration buffer, followed by a linear gradient of 0 - 0.4 M potassium chloride for 20 min and a 20 min wash with 0.4 M potassium chloride (in equilibration buffer). The flow-rate was 1 ml/min and 0.5 ml fractions were collected and assayed for GST activity (Singh *et al*, 1986).

Purity of erythrocyte GST ρ was determined by SDS-PAGE (Laemmli, 1970). Protein concentrations were determined by the Bio-rad method since both

β-mercaptoethanol and GSH were compatible with this assay (Bradford, 1976; Bio-rad instruction manual).

All buffers containing DTT and GSH were made up in degassed water immediately before use. Buffers required for the HPLC were degassed and passed through 0.22 μm filters. β-mercaptoethanol at the required concentrations did not affect the pH of the relevant buffers (own observation) and was only added to these buffers before use.

2.8 Enzyme Incubations.

2.8.1 Incubations with Inhibitors.

The enzyme was usually incubated with halocarbons in 500 μl volumes containing enzyme (variable volume), inhibitor (40 μl) and 35 mM Tris.HCl, pH 8.2, for 15 min at 25 °C. When present, GSH (40 μl) and EDTA (20 μl) were added to the incubations at final concentrations of 10 mM and 4.2 mM, respectively. Incubation mixtures were kept on ice until the enzyme was added. Enzyme activity was measured at 0 min incubation and after a 15 min incubation at 25 °C. The reaction was then terminated by placing the incubation mixtures on ice at 4 °C prior to assay.
The enzyme was added to the incubations at a sufficient concentration to give an absorbance change of 0.05 per min in the standard CDNB assay (50 μl diluted enzyme in a total assay volume of 3 ml).

2.8.2 Inhibitor Solutions.

Inhibitors (halothane, isoflurane, methoxyflurane, enflurane, ethylene dibromide) were dissolved in ethanol to a concentration of 0.5 M, with a final concentration of 40 mM in the incubations (40 μl/500 μl incubation; 8% ethanol).
2-Chloro-1,1-difluoroethylene (a gas) was dissolved in 10 ml cold ethanol (4 °C) in a gas-tight stoppered test tube. Solutions were weighed and concentrations calculated (by the mass difference).

2.8.3 Bilirubin Incubations.

GST isoenzymes were incubated with bilirubin or NaOH and, as indicated, other components, in 20 mM potassium phosphate buffer containing 0.1 M NaCl, pH 6.5, at 25 °C in cuvettes without shaking. Incubation times and order of addition of components are shown with the relevant results. Enzyme activity was measured in cuvettes following consecutive addition of 1.0 mM CDNB and 2.5 mM GSH.
GSH (50 mM) and bilirubin (2.1 mM in 10 mM NaOH; protected from light) were prepared daily, and CDNB (30 mM; protected from light) was prepared weekly.

2.8.4 Incubations with Microperoxidases.

GST ρ was incubated with microperoxidases in 0.1 M phosphate buffer (pH 6.5) for 15 min at room temperature (24 ± 2 °C) before assay of enzyme activity in a Multistat centrifugal analyzer.

2.9 Reversible Activation/Inhibition by Halothane and DBE.

The reversible activation and/or inhibition by halothane and DBE was measured using the standard CDNB assay previously referred to. Either halothane or DBE was mixed with 0.1 M phosphate buffer, pH 6.5, in a stoppered test tube. The buffer (2.75 ml) was added to a cuvette and CDNB (20 μl) added. The cuvettes were equilibrated at 25 °C, and GSH (150 μl) was added before initiation of the reaction with enzyme (50 μl).
In experiments with varied CDNB concentrations (0.028 - 0.27 mM) the GSH concentration was kept constant at 1.0 mM; In experiments with varied GSH concentrations (0.132 - 1.43 mM), the concentration of CDNB was maintained at 0.27 mM.
Halothane and DBE (0.5 M stock solution in ethanol), CDNB and GSH solutions were prepared daily. The enzyme was diluted in buffer to give an absorbance change of approximately 0.07 per min at 340 nm at the highest concentration of substrates.

2.9.1 Reversibility of Activation/Inhibition by Dilution.

Halothane was added to the buffer to give a final concentration of 22.4 mM and the solution equilibrated to 25 °C. In order to determine enzymic activation, CDNB and GSH were added to cuvettes containing the halothane mixture and the enzyme added immediately before the kinetics were started. To evaluate reversibility the enzyme was added to smaller volumes of halothane/buffer and diluted immediately with a solution of buffer containing CDNB and GSH.

2.10 Synthesis of S-(2,4-Dinitrophenyl)GSH.

CDNB (30 mM) was dissolved in 50% aqueous ethanol and a stoichiometric equivalent of GSH added over a period of 40 min with continuous stirring. The pH was maintained between 7 and 8 during the reaction by addition of NaOH. The reaction was monitored spectrophotometrically at 340 nm. On completion of the conjugation reaction the volume was reduced (± 50%) under vacuum and unconjugated CDNB removed by 3 extractions with diethyl ether. Ethanol was added to the solution to faint turbidity before storage at 4 °C for 48 hours. An oily precipitate was dissolved in water and chromatographed on a Sephadex G-15 column (2.5 cm x 30 cm) equilibrated with H_2O. GSH-free fractions containing S-(2,4-dinitrophenyl)GSH were lyophilized and stored (Schramm *et al*, 1984).

2.11 Preparation of Microperoxidases.

The conditions for the preparation of MP-6, MP-8, MP-9, and MP-11 are detailed below (Table 2.2). Samples were usually incubated for 24 hours at 40 °C and lyophilized before being redissolved in 0.1% TFA for purification by HPLC.

Table 2.2: Conditions for the preparation of MP-6, MP-8, MP-9 and MP-11.

Microperoxidase	Substrate[a]	Enzyme	Enzyme[a] (amount)
6	cytochrome c	nagarse	4 mg
8	MP-11	trypsin	37.5 mg
9	cytochrome c	trypsin	5 mg
11	cytochrome c	pepsin[b]	64 mg

[a] The substrates and enzyme were dissolved in 0.1 M ammonium phosphate buffer, pH 8.5 (20 ml).
[b] Pepsin was added in 2 batches: 32 mg at 0 min and 32 mg 15 min later.
(Peterson *et al*, 1980, 1983; Baba *et al*, 1969; Plattner *et al*, 1977).

2.11.1 Purification of Microperoxidases by HPLC.

Microperoxidases were purified on a MicroBondpak C-18 reverse phase HPLC column using 30% acetonitrile/0.1% TFA as the aqueous phase with a flow rate of 2 ml/min. Elution was monitored at 398 nm (heme moieties) or 220 nm (peptides). Heme peptides were collected and purity confirmed by rechromatography on the HPLC column. Retention times were determined by the use of microperoxidases obtained commercially. Purified samples were lyophilized and stored at 4 °C.

2.11.2 Extinction Coefficients.

The microperoxidases are known to aggregate, with a concomitant decrease in the extinction coefficient. The extinction coefficients for MP-8 and MP-9 are 1.57×10^5 (monomer) and 1.21×10^5 (dimer) $M^{-1}.cm^{-1}$ in 0.1 M phosphate buffer, pH 7.0 (Baldwin *et al*, 1987). Microperoxidase concentrations were therefore measured at absorbances below 0.15 (*ie* $\leq 1\ \mu M$) at the Soret peak (approximately 396 nm). The dimer concentration in this region was assumed to be 10% and an extinction coefficient of $1.53 \times 10^5\ M^{-1}.cm^{-1}$ was used for MP-8 and MP-9. MP-11 was dissolved in 0.1 M phosphate (pH 7.0) and a small aliquot ($\leq 100\ \mu l$/ 3 ml) diluted in 10 mM HCl (pH 2 - 2.5) and the absorbance measured at the Soret peak ($\epsilon_{394\ nm} = 178\ mM^{-1}.cm^{-1}$) (Peterson *et al*, 1983).

2.11.3 Binding of the Microperoxidases to GST p.

Microperoxidase binding was measured spectrally at 396 nm (25 ± 0.2 °C) using a Varian Techtron 635 spectrophotometer. Data was transferred to a Hewlett Packard 85 computer through a Hewlett Packard 3438A digital multiplier and a print-out obtained for analysis.

2.12 Statistics and Calculations.

In general, results are expressed as the mean ± standard deviation.
Student's t-test was used to determine a significant difference between means; $P < 0.01$ was assumed to be significant, while $P < 0.001$ was highly significant. Student's t-test was performed on Epistat (Gustafson, 1985).
In the chapter relating to inhibition by halothane (Chapter 3) the data were also analyzed according to the following method as proposed by the Institute of Biostatistics of the South African Medical Research Council: The mean and standard deviation were computed for 0 min samples separately for controls and control + inhibitor. For each datum value the 0 min mean (of the corresponding experiment) was subtracted and this value divided by the relevant 0 min standard deviation. The same process was repeated for the control + inhibitor experiments. A two-way analysis of variance was then carried out on this data with 0 min and 15 min as the levels of one factor, and control and control + inhibitor as the levels of the other factor. The comparison between control and control + inhibitor was therefore tested.
No difference in statistical parameters was found between the latter method and Student's t-test, although the analysis of variance was more complicated to perform.

All model fitting to relatively simple equations (Michaelis-Menten equation; first and second order decay curves) was performed on a non-linear least squares regression program with no weighting of the data (Enzfitter, Elsevier-Biosoft, Cambridge, U.K.). Fitting of complex kinetic mechanisms was accomplished on a statistical non-linear regression program, BMDPAR, using the inverse of the variance as a weighting factor (BMDP Statistical Software, University of California, USA). The relevant equations are shown in the results section.

Halothane: Inhibition and Activation of Rat Hepatic GST.

3.1 The Effect of Halothane Anesthesia on Rat Hepatic GST Activity *in vivo*.

A single anesthesia of rats with halothane (1.25 MAC for 3 h) had no significant inhibitory effects on rat cytosolic GST activity (Table 3.1). In contrast, multiple halothane anesthesia (1.25 MAC for 1 h on three alternative days) initially decreased and subsequently increased GST activity, reaching a minimum at 10 days after the first anesthesia and a maximum after 25 days (Table 3.2). The effect appeared to be isoenzyme specific with DCNB activity (specific for subunit 3) markedly affected, while the effect on *trans*-4-phenyl-3-buten-2-one activity (specific for subunit 4) was not as dramatic. GSH peroxidase activity, as measured with cumene hydroperoxide, showed the same trend as GST with CDNB as substrate. However, although this substrate is relatively specific for GST subunits 3, 4 and 5, it is also a substrate for the selenium-dependent GSH peroxidases.

Table 3.1: Effect of a single halothane anesthesia on rat hepatic GST activity *in vivo*.

	Time after anesthesia (days)					
	1	4	10	15	25	32
Activity	106 ± 9	98 ± 6	116 ± 8[a]	84 ± 6	97 ± 6	121 ± 9

Rats were treated with 1.25 MAC halothane for 3 hours on day 1. Specific activity of cytosolic GST was measured with CDNB. Activity (%) is relative controls exposed to air under identical conditions (N = 3 - 4 for each determination).
[a] Significantly different from controls ($P < 0.01$).
No significant differences were detected at $P < 0.001$.

Table 3.2: Effect of multiple halothane anesthesias on rat hepatic GSH S-transferase and GSH peroxidase activity.

		% Activity relative to controls					
		Time after first halothane anesthesia (days)					
Assay	Activity per	5	10	16	20	25	30
CDNB	mg protein	94 ± 3	64 ± 2[a]	93 ± 3	108 ± 4	153 ± 2[a]	111 ± 2[a]
	g liver	94 ± 3	70 ± 2[a]	89 ± 3[b]	92 ± 3	114 ± 2[a]	125 ± 2[a]
	(N)	(6)	(4)	(6)	(6)	(6)	(6)
DCNB	mg protein	89 ± 2[a]	66 ± 1[a]	90 ± 1[a]	103 ± 2	133 ± 1[a]	102 ± 2
	g liver	90 ± 2[b]	72 ± 1[a]	85 ± 1[a]	87 ± 2[a]	99 ± 1	115 ± 3[a]
	(N)	(4)	(6)	(6)	(6)	(6)	(6)
trans-4-phenyl-3-buten-2-one	mg protein	95 ± 7	90 ± 1[a]	115 ± 6[a]	104 ± 2	109 ± 2[b]	95 ± 3[a]
	g liver	95 ± 7	98 ± 2	110 ± 6	88 ± 1[a]	82 ± 4[a]	107 ± 3[b]
	(N)	(6)	(6)	(6)	(8)	(7)	(8)
Cumene hydroperoxide	mg protein	101 ± 3	84 ± 2[a]	99 ± 1	122 ± 3[a]	185 ± 7[a]	107 ± 1[a]
	g liver	102 ± 3	92 ± 3[b]	95 ± 1[b]	103 ± 3	138 ± 5[a]	121 ± 1[a]
	(N)	(6)	(6)	(6)	(6)	(6)	(6)

Groups of 9-15 rats were exposed to 1.25 MAC halothane or medical air for 1 hour on days 1, 3 and 5. Results are from one experiment, with each time point representing Ndeterminations on the pooled hepatic cytosol from 3 control or 3 anesthetized rats. Similar results for days 5, 10 and 16 were obtained in a separate experiment.

Specific activities (μmol/min/mg protein) for control rats were as follows: CDNB, 1.0; DCNB, 0.055; *trans*-4-phenyl-3-buten-2-one, 0.013; Cumene hydroperoxide, 0.17.

[a] Differs significantly from corresponding control values, $P < 0.001$

[b] Differs significantly from corresponding control values, $P < 0.01$

3.2 Inhibition of GST Activity *in vitro*.

Incubation of the mixture of GST isoenzymes with four volatile anesthetics (Table 3.3) significantly inhibited enzyme activity in a time-dependent manner in the absence of GSH. A metabolite of halothane, 1-chloro-2,2-difluoroethylene (16 - 57 mM) did not show significant concentration-dependent inhibition of the enzyme, with activities varying from 80% - 104% relative to controls. Purification of the mixture of GST isoenzymes in the presence of DTE, a thiol reagent, did not have any effect on inhibition (Table 3.3).

Table 3.3: Time-dependent inhibition of the mixture of rat GSTs by volatile anesthetics and DBE *in vitro*.

	% CDNB activity after 15 min incubation (N)	
Anesthetic (mM)	Control	+ Anesthetic
Halothane (40)[a]	91 ± 5 (18)	70 ± 1 (18)[c]
Isoflurane (40)[a]	93 ± 7 (12)	76 ± 4 (12)[c]
Enflurane (40)[a]	93 ± 7 (12)	70 ± 10 (12)[c]
Methoxyflurane (40)[a]	93 ± 7 (12)	65 ± 5 (12)[c]
Halothane (40)[b]	92 ± 5 (6)	78 ± 2 (8)[c]
Isoflurane (40)[b]	92 ± 5 (6)	74 ± 2 (8)[c]
DBE (40)[b]	92 ± 5 (6)	45 ± 4 (8)[c]

[a] Incubations contained the mixture of rat liver GSTs (*ca* 0.3 units/ml), and a solution of ethanol or anesthetic in ethanol in 35 mM Tris.HCl, pH 8.2, at 25 °C.
[b] The enzyme was purified in buffer containing 0.2 mM DTE.
[c] Differs significantly from value for control, $P < 0.001$.
Activity (%) is relative to identical zero time samples.

Inhibition of the GST mixture by halothane followed pseudo first-order kinetics (Fig. 3.1) in the absence of GSH. The inhibition was essentially complete after 30 min. The observed first-order rate constant for inhibition was $0.0022 \pm 0.0002\ s^{-1}$ ($t_{1/2}$ = 5.4 min).

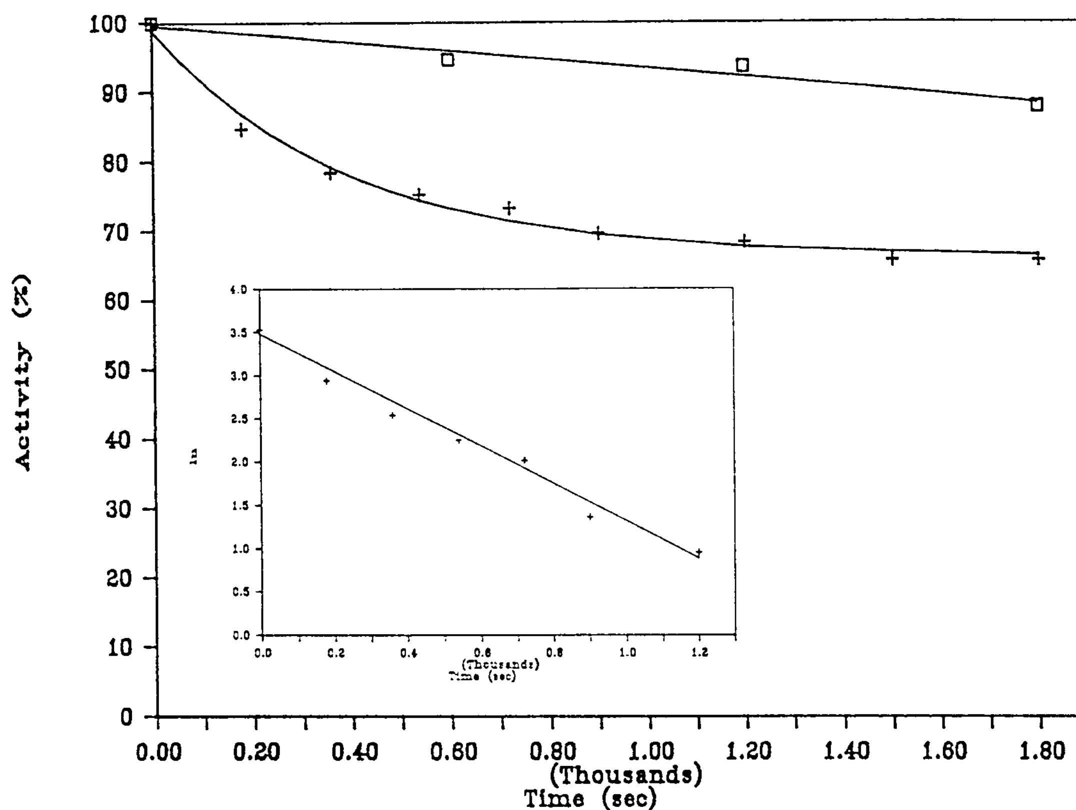


Fig. 3.1: Time-dependent inhibition of GST activity by halothane. The GST mixture (*ca* 0.44 units/ml) was incubated with 4 mM EDTA and 35 mM Tris.HCl, pH 8.2, in the absence (□) and presence (+) of 40 mM halothane at 25 °C. Activity (%) is relative to a zero time sample in the absence of halothane. Inset: $\ln(A_\infty - A_t)$ vs time.

[a] Differs significantly from zero time sample, $P < 0.05$

[b] Differs significantly from zero time sample, $P < 0.01$

Halothane inhibition of the mixture of GST isoenzymes was concentration-dependent with apparent half-maximal inhibition at concentrations of greater than 15 mM halothane (Fig. 3.2). However, assays were not carried out in the linear region of the time-dependent inhibition process (Fig. 3.1).

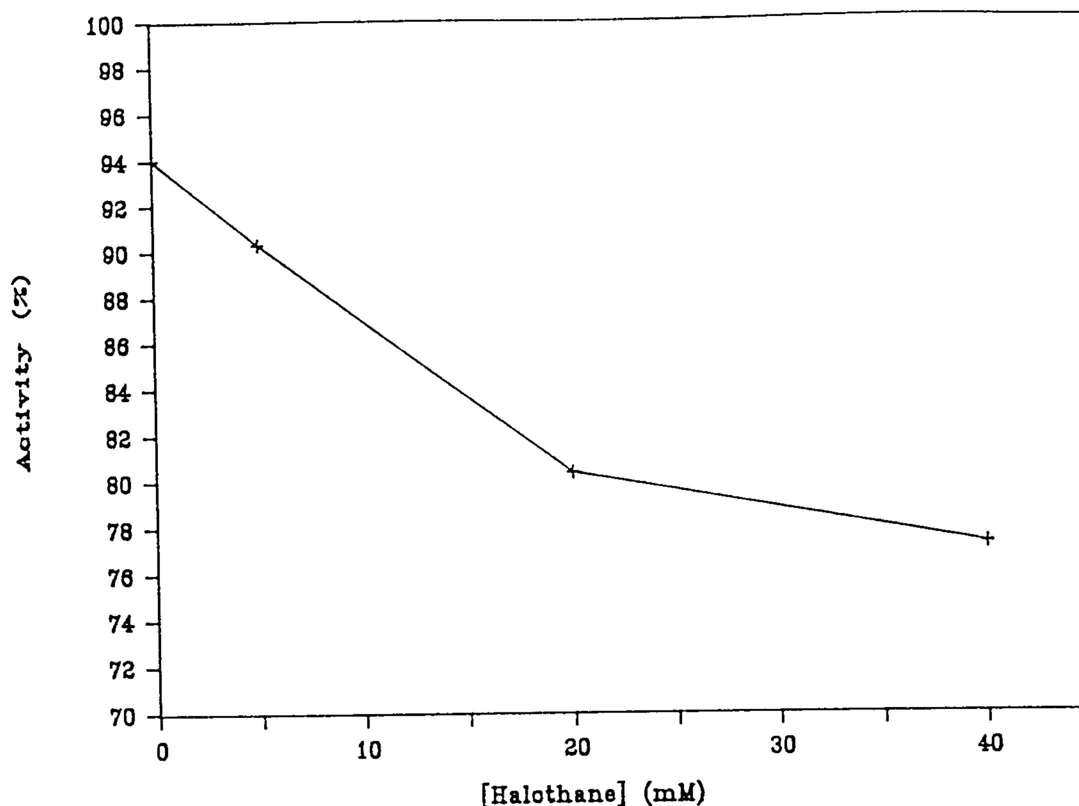


Fig. 3.2: Concentration-dependence of halothane inhibition. The GST mixture (*ca* 2.0 units/ml) was incubated with 10 mM GSH, 4 mM EDTA, and halothane in 35 mM Tris.HCl, pH 8.2, for 15 min at 25 °C. Activity (%) is relative to a zero time sample in the absence of halothane.
[a] **Differs significantly from zero time control, $P < 0.01$**

Halothane inhibition of rat GST activity was found to be isoenzyme specific (Table 3.4). Isoenzymes containing subunits 1 and 2 were not inhibited by halothane, while isoenzymes containing subunits 3 and 4 were significantly inhibited.

In contrast to the rat isoenzymes, human placental isoenzyme π was not inhibited by either anesthetic agents or DBE (Table 3.5).

Table 3.4: Time-dependent inhibition of rat GST isoenzymes by halothane.

	% CDNB activity after 15 min incubation	
Isoenzyme	Control	+ Anesthetic
1-1	N.D.[a]	94 ± 3
1-2	95 ± 3	89 ± 6
3-3	109 ± 3	43 ± 3[b]
3-4	85 ± 4	62 ± 4[b]
4-4	80 ± 1	45 ± 12[b]

Incubation mixtures contained pure rat GST isoenzymes (*ca* 0.3 units/ml), ethanol or halothane in ethanol (40 mM), and 10 mM GSH in 35 mM Tris.HCl, pH 8.2, at 25 °C (N = 3 - 4 for each analysis).
Activity (%) is relative to identical zero time samples.
[a] N.D. Not determined.
[b] Differs significantly from value for control, P < 0.01.

Table 3.5: Time-dependent inhibition of human placental GST π *in vitro*.

	% CDNB activity after 15 min incubation (N)	
Anesthetic (mM)	Control	+ Anesthetic
Halothane (40)	95 ± 4 (8)	95 ± 6 (8)[a]
Isoflurane (40)	95 ± 4 (8)	90 ± 3 (8)[a]
Enflurane (40)	95 ± 4 (8)	92 ± 4 (8)[a]
DBE (40)	95 ± 4 (8)	92 ± 3 (8)[a]

Incubation mixtures contained the mixture of human placental GST (*ca* 0.3 units/ml enzyme stored in 50 mM Tris.HCl containing 10 mM GSH, pH 9.6), and a solution of ethanol/anesthetic in ethanol in 35 mM Tris.HCl, pH 8.2, at 25 °C.
Activity (%) is relative to identical zero time samples.
The placental enzyme was purified in our laboratory by R.D. Goold.
[a] No significant differences were detected.

3.3 Prevention of Halothane Inhibition by Cytosol *in vitro*.

The addition of diluted cytosol (0.02 mg protein)* or albumin (200 mg) to incubations of the GST mixture prevented inhibition by halothane, although albumin significantly activated the enzymic activity (data not shown).

Incubations of cytosol with inhibitors of GST activity showed that only CDNB significantly affected enzyme activity in the cytosol (Table 3.6). Neither the *in situ* perfusion of rat livers nor the dialysis of cytosol resulted in inhibition of cytosolic GST activity by halothane (Table 3.7).

Table 3.6: Incubations of cytosol with GST inhibitors.

Addition	(mM)	Percentage activity (N) Control	+ Halocarbon
Halothane	(40)	98 ± 5 (12)	102 ± 4 (12)
DBE	(37)	98 ± 5 (12)	105 ± 4 (4)
CDNB	(1.2)	98 ± 5 (12)	75 ± 4 (8)[a]

Cytosolic GST (*ca* 0.4 units/ml) was incubated with the above inhibitors in 35 mM Tris.HCl (pH 8.2) for 15 min at 25 °C. Inhibitors were dissolved in ethanol to give a concentration of 8% ethanol in the incubation. CDNB activity (%) is relative to identical zero time samples.
[a] Significantly different from controls containing ethanol only, $P < 0.001$.

* Footnote: Cytosol contributed less than 10% of the total GST activity in the incubations.

Table 3.7: Effect of perfusion and dialysis on the time-dependent inhibition of cytosolic GST activity by halothane.

Treatment of rat	Additions to incubation	CDNB activity (%)		
		Time of dialysis (h)		
		0	1	24
None	Ethanol	95.8 ± 5.0 (8)	94.7 ± 2.6 (8)	94.4 ± 3.4 (8)
	Halothane	96.6 ± 3.4 (8)	92.7 ± 1.9 (8)	98.9 ± 1.9 (8)
Liver perfusion	Ethanol	94.7 ± 3.6 (8)	97.5 ± 1.8 (8)	97.9 ± 4.4 (8)
	Halothane	88.0 ± 4.3 (12)	97.6 ± 6.1 (7)	99.7 ± 3.3 (8)

Reaction mixtures containing cytosolic GST (*ca* 0.5 units/ml), 4 mM EDTA, and ethanol (8%) or halothane (40 mM in ethanol) in 35 mM Tris.HCl, pH 8.2, were incubated at 25 °C for 15 min. Samples were dialyzed at 4 °C versus 10 mM Tris.HCl, pH 7.8, for 22 hours. Rat livers were perfused *in situ* with 10 mM Tris.HCl, pH 7.8, at 4 °C. Activity (%) is relative to identical zero time samples.

3.4 Reversal of Halothane Inhibition *in vitro*.

As a result of the volatility of halothane, we attempted to reverse inhibition by removing the anesthetic from incubations by bubbling with nitrogen. The GST mixture was incubated with ethanol or halothane in the presence of 10 mM GSH for 15 min, and the samples were then divided, one aliquot exposed to air for 5 min while the second aliquot was bubbled with nitrogen. Bubbling with nitrogen resulted in an approximately 25% loss in activity. Reversal of halothane inhibition was not indicated as the difference between control and halothane samples was still approximately 13%. These results are, however, inconclusive in view of the loss of activity incurred by the nitrogen bubbling procedure.

3.5 Metabolism of Halothane and DBE *in vitro*.

Halothane was not metabolized measurably by cytosolic GST ($\leq$ 0.2 nmol), as determined by glutathione metabolism (Fig. 3.3). In contrast, DBE was significantly conjugated with GSH under identical conditions (3.8 nmol.min^{-1}.mg protein^{-1} during the first 30 min of incubation).
Metabolism was measured with cytosol since GST activity was not inhibited by either halothane or DBE under these conditions (see above).

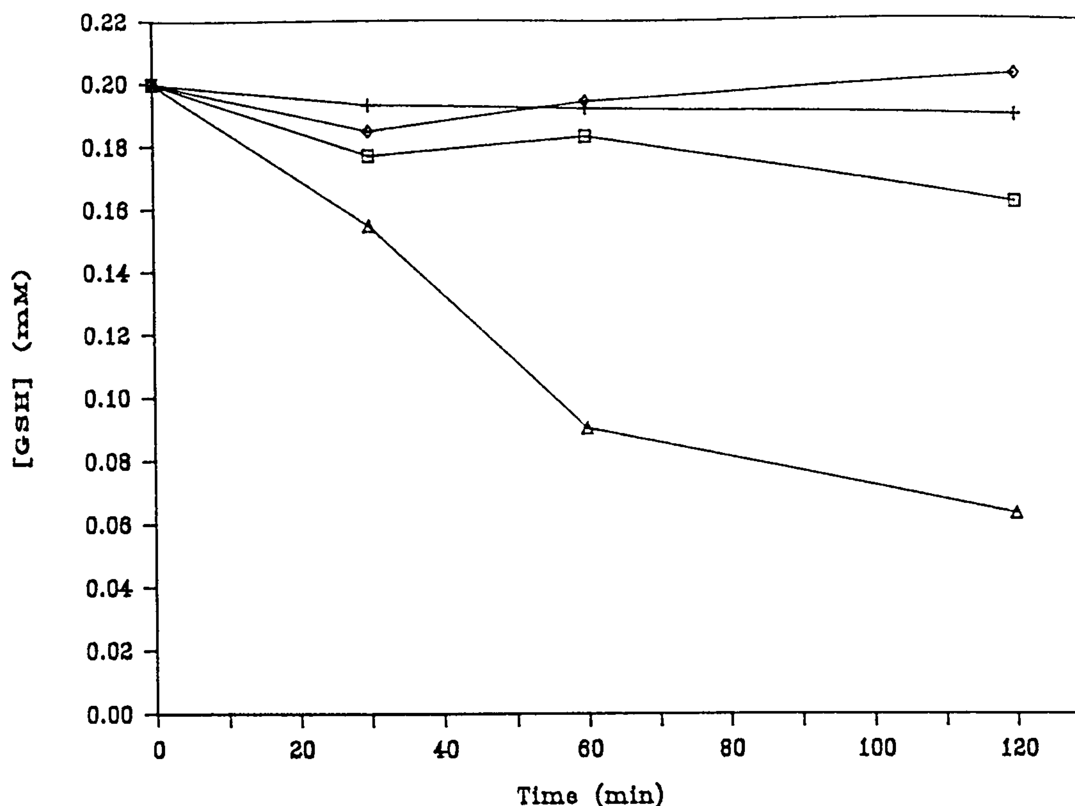


Fig. 3.3: Metabolism of halothane and DBE by cytosolic GST in the presence of GSH. Halothane + GSH (□); Halothane + GSH + GST (+); DBE + GSH (◇); DBE + GSH + GST (△).
Halothane and DBE (20 mM) were incubated with 0.2 mM GSH in the presence and absence of cytosol (± 0.04 mg protein) in 35 mM Tris.HCl, pH 8.2, at 25 °C. Aliquots of the incubations were removed at the specified time points and lyophilized. GSH concentrations were determined after dilution with 10 mM HCl using the GSH assay effective in the micromolar concentration range.

Attempts to measure halothane metabolism by bromide release were not successful as bromide levels in incubations of the GST mixture were lower than the detection limit (5 μM Br^-) of the bromide-selective electrode used. The enzyme was incubated with 20 mM halothane, 4 mM EDTA and 5 mM GSH in 35 mM Tris.HCl, pH 8.2, at 25 °C for 15 min (data not shown).

3.6 Reversible Activation by Halothane *in vitro*.

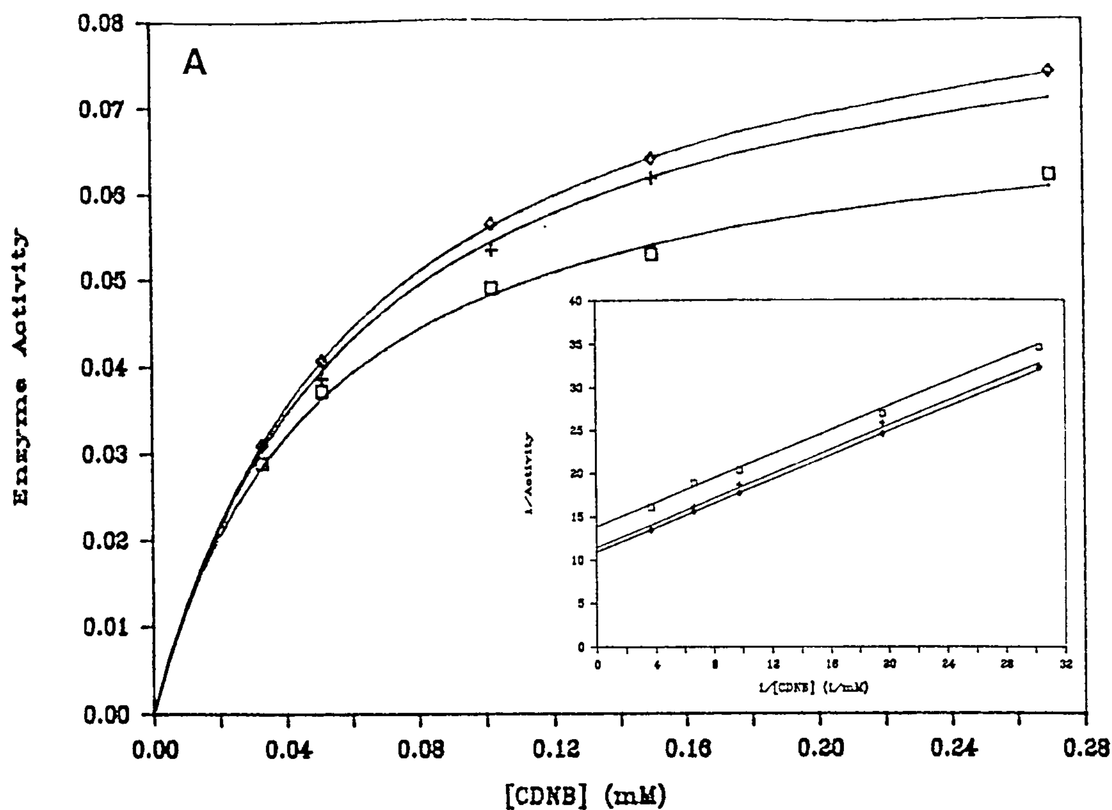
The reversible effect of halothane on GST activity was measured at various concentrations of the substrates CDNB and GSH in the presence of halothane using a modification of the standard CDNB assay: Phosphate buffer (pH 6.5) was pre-equilibrated with halothane and CDNB at 25 °C in cuvettes before the addition of GSH and enzyme immediately before spectral tracings were begun.

Activation of the GSTs was rapid and complete before spectral tracings were begun (*ie* $t_{1/2} \leq 5$ sec) and was readily reversible upon dilution of halothane in reaction mixtures (Table 3.8).

Activation was related to halothane, CDNB and GSH concentrations. Cytosolic transferases were consistently activated by halothane (Fig. 3.4). Of the purified isoenzymes, only isoenzyme 1-2 was significantly activated by halothane *in vitro* (Fig. 3.5). Isoenzymes 1-1 and 3-3 were not significantly affected by halothane over a range of CDNB concentrations* (Fig. 3.6).
Dilution of halothane from 22.4 to 11.2 and 7.8 mM in the presence of cytosolic GST decreased halothane-dependent activation from 117 ± 1% to 113 ± 1% and 104 ± 1%, respectively (relative to samples containing ethanol treated in an identical manner).

---

* Footnote: Although the lines fitted by linear regression are different in the presence and absence of halothane, the values obtained at the different CDNB concentrations were not significantly different.



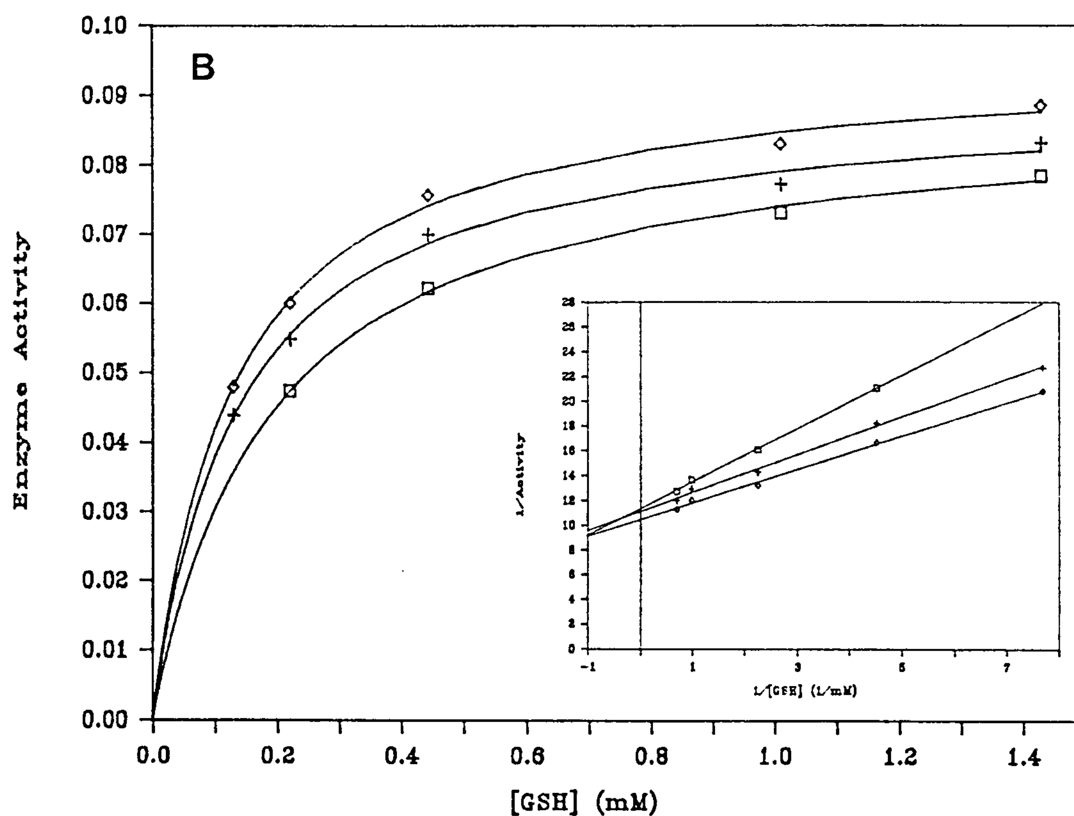


Fig. 3.4: Reversible activation of cytosolic GST activity by halothane with respect to CDNB (A) and GSH (B) variation. Ethanol (□); 11.2 mM halothane (+); 22.4 mM halothane (◇).
Insets show the double-reciprocal plots of the relevant data. Curves were fitted to Michaelis-Menten equation. Activity is shown as $\Delta A_{340\ nm}$/min.

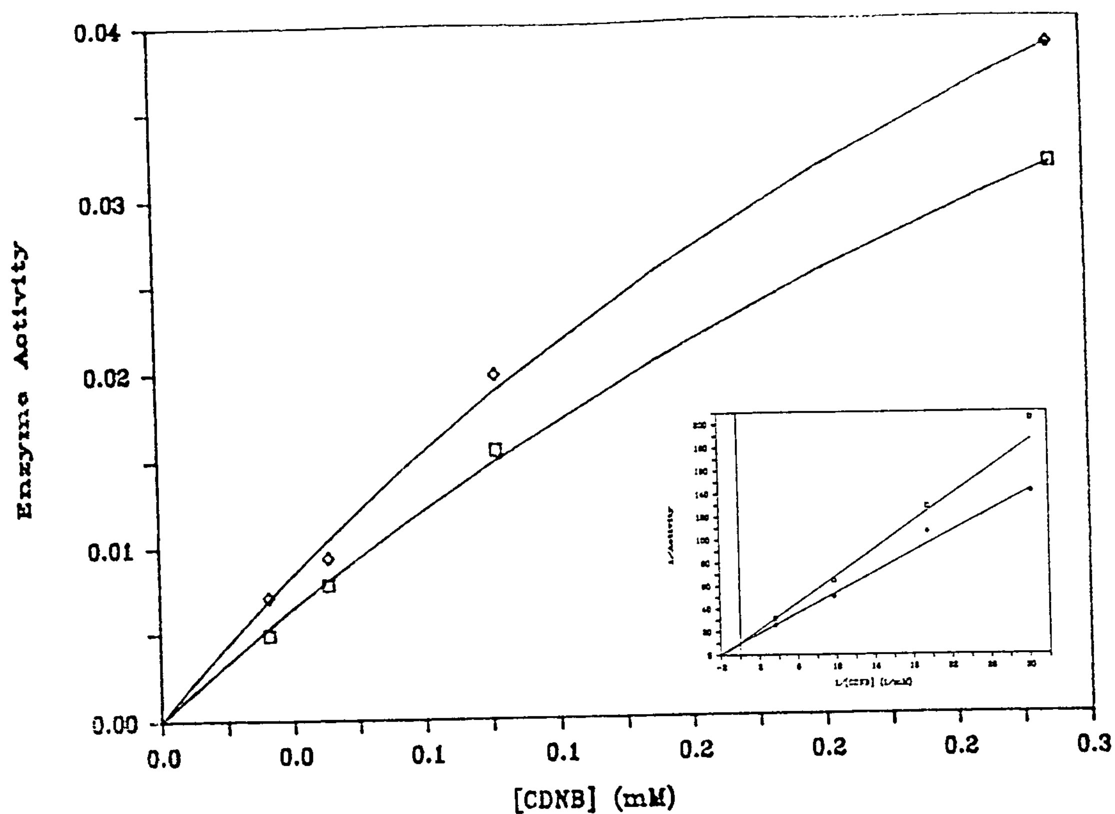


Fig. 3.5: Reversible activation of GST 1-2 by halothane as a function of CDNB concentration. Ethanol (□) and 22.4 mM halothane (◇).
Insets show the double-reciprocal plots of the relevant data. Curves were fitted to Michaelis-Menten equation. Activity is shown as ΔA_{340} nm/min.

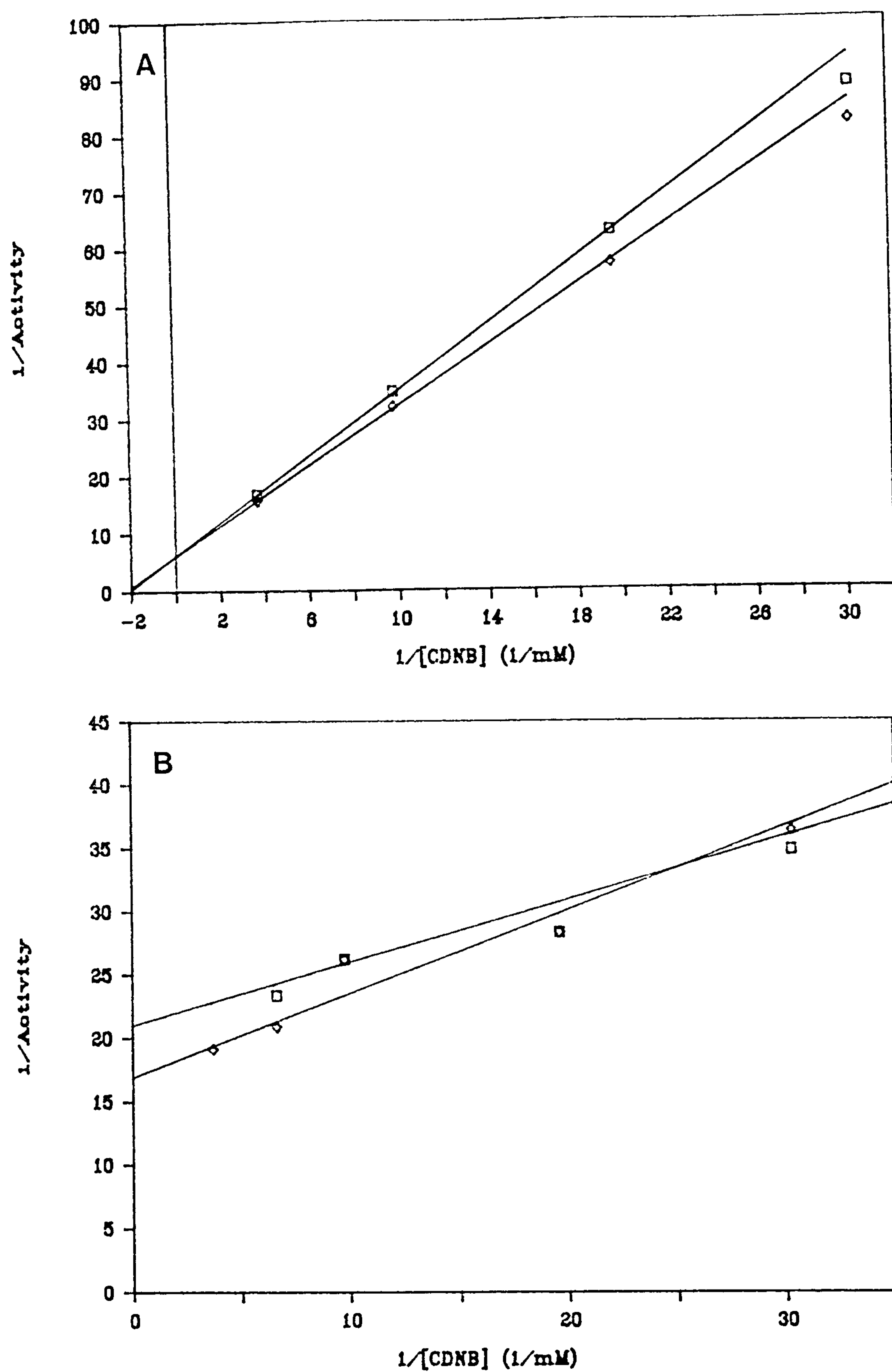


Fig. 3.6: Reversible activation of GST 1-1 (A) and 3-3 (B) by halothane as a function of CDNB concentration. Ethanol (□) and 22.4 mM halothane (◇). Insets show the double-reciprocal plots of the relevant data. Curves were fitted to Michaelis-Menten equation. Activity is shown as $\Delta A_{340\ nm}$/min.

Miscellaneous Results:

3.7 Purification of Rat GSH S-Transferases.

The mixture of rat GSH S-transferases was purified by affinity chromatography on either S-hexyl GSH- or GSH-Sepharose with two chromatographic steps on Sephadex G-25. The specific activity of the final material was consistently found to be within the range of 15 - 25 μmol.min^{-1}.mg protein^{-1}. It was found that the two steps on Sephadex G-25 consistently decreased enzyme activity by approximately 30% per chromatographic run, consequently the effect of dialysis on GST activity was assessed.

Chromatography on Sephadex G-25 (3.9 cm x 17 cm) was found to decrease cytosolic GST activity to 68 ± 5% of the total activity loaded, whereas dialysis (22 hours) did not significantly decrease activity (96 ± 6%). The time course of GSH dialysis indicated that more than 95% of the GSH was removed after 24 hours (Fig. 3.7). GSH concentrations were decreased to 10.2% and 5.7% upon chromatography and dialysis, respectively.

3.7.1 Effect of DTT on GST Activity.

The addition of dithiothreitol (1 mM final concentration) to the enzyme before incubation at 4 °C slightly increased enzyme activity after 22 hours: At 2, 4 and 22 hours after dilution of enzyme activity into 1 mM DTT, GST activity was 92%, 97% and 107% of the activity at zero time.

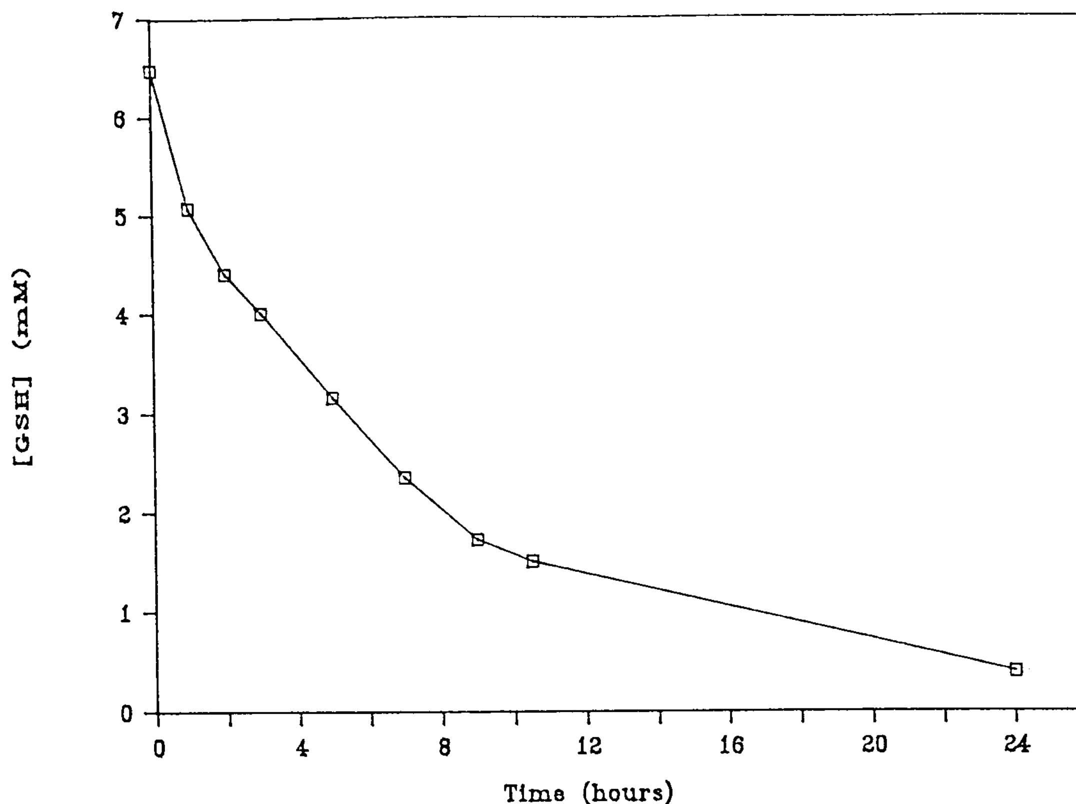


Fig. 3.7: Time course of GSH dialysis. GSH (50 ml in H_2O) was dialyzed against one change of H_2O (4000 ml) after 5 hours. Aliquots were removed and diluted with H_2O before GSH concentrations were determined with an assay effective in the millimolar range (see Section 3.12).

3.8 Effect of Solvents on Enzyme Activity.

The electrophilic substrates generally used to assay for GST activity (*eg* CDNB) are relatively insoluble in water and must therefore be dissolved in organic solvents before addition to the GST assay buffer. Since various solvents have been reported to inhibit enzyme activity (Aitio & Bend, 1979), the effect of solvents on GST activity in the assay system and upon incubation was investigated. Acetone caused the smallest apparent initial decrease in activity (Table 3.8), but had a greater inhibitory effect than ethanol when incubated with the enzyme for 15 min at pH 8.2 (Table 3.9).
Previous studies have shown that the conjugation of DCNB with GSH in rat liver cytosol is markedly inhibited (> 35%) by solvents such as acetonitrile, dioxane, ethanol and methanol (3.3%, v/v). However, DMSO, dimethylformamide, dioxolane and tetrahydrofuran were among the solvents which were less inhibitory

($\leq$ 20% inhibition relative to controls containing 0.33% DMSO) (Aitio & Bend, 1979).

Table 3.8: Effect of different solvents on GST activity.

Solvent	% (v/v)	Activity (%)
None	-	100
Ethanol	5	77
	10	68
Methanol	5	68
	10	47
DMSO	5	84
	10	77
Acetone	5	89
	10	85
	20	73
Acrylonitrile	5	22

The solvents were added to the standard CDNB assay (3.3% ethanol) and enzyme activity assayed following addition of GST.
The partially purified mixture of rat GST was used in these studies.

Table 3.9: Effect of incubation with acetone and ethanol on GST activity.

Addition	Time (min)	Activity (%)
Ethanol (8%)	15	89
Acetone (8%)	2	96
	5	83
	15	53

The enzyme was incubated with the above solvents (40 μl per 500 μl incubation) for 15 min at 25 °C and activity measured using the CDNB assay (3.3% ethanol) for comparison with identical zero-time samples.
The partially purified mixture of rat GST was used in these studies.

3.9 Linearity of GST Assays on Beckman Spectrophotometer.

The initial rate CDNB assay is linearly-dependent on enzyme concentration for absorbance changes below 0.05 $A_{340\ nm}$/min (Fig. 3.8). Spectral traces deviate from straight line behavior after 1 min (data not shown), requiring initial rates to be determined in the region $0 < t \leq 1$ min.

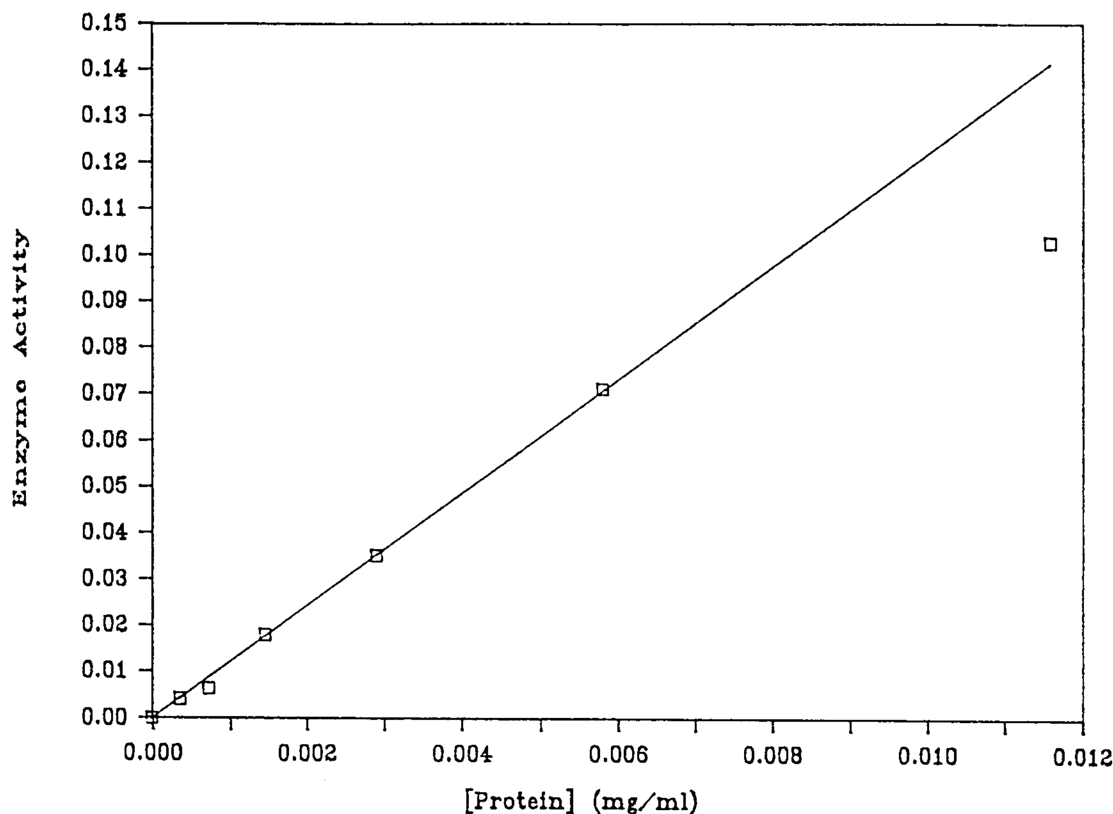


Fig. 3.8: Linearity of the CDNB assay on a Beckman UV 5230 spectrophotometer. The assay was performed in 3 ml glass cuvettes (1 cm pathlength) at 25 °C in 0.1 M phosphate buffer, pH 6.5, with 50 μl diluted enzyme (rat liver cytosol, 1.12 ± 0.18 μmol.min^{-1}.mg protein^{-1}), 1.0 mM GSH and 1.0 mM CDNB (3.3% ethanol). Activity is shown as $\Delta A_{340\ nm}$/min.

3.10 Linearity of the Cumene Hydroperoxide Assay.

The peroxidase assay utilizing cumene hydroperoxide as substrate was shown to linear at less than 0.05 $A_{340\ nm}$/min for one min (Fig. 3.9).

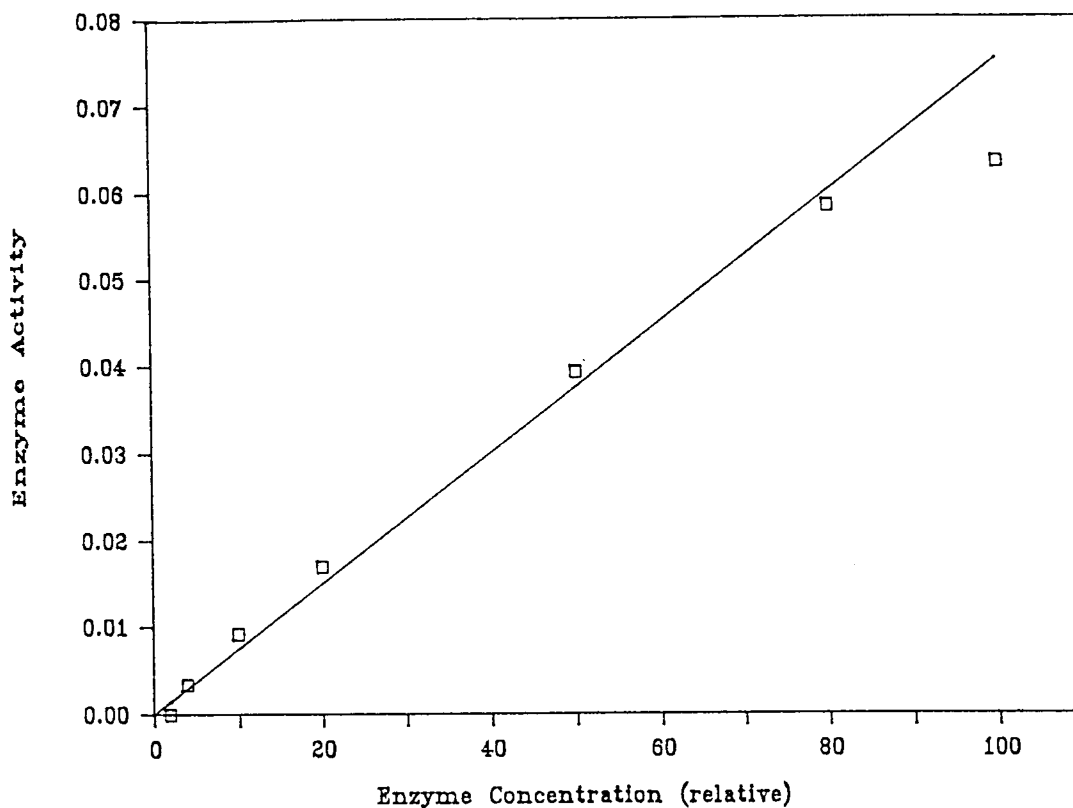


Fig. 3.9: Linearity of the cumene hydroperoxide peroxidase assay on a Beckman UV 5230 spectrophotometer. The assay was performed in 1 ml cuvettes (1 cm path length) at room temperature and 10 μl diluted enzyme added before activity measured at 340 nm for one min.
The partially purified mixture of rat GST (0.275 ± 0.025 $\mu mol.min^{-1}$) was used in this experiment.

3.10 Bromide Electrode.

A standard curve for the bromide electrode used as a reference in the determination of the extent of halothane metabolism is shown in Fig. 3.10. The electrode exhibited a linear millivolt response to Br^- concentration between 5 μM and 50 μM. The lower limit of sensitivity of this method was 5 μM bromide (as per manufacturer).

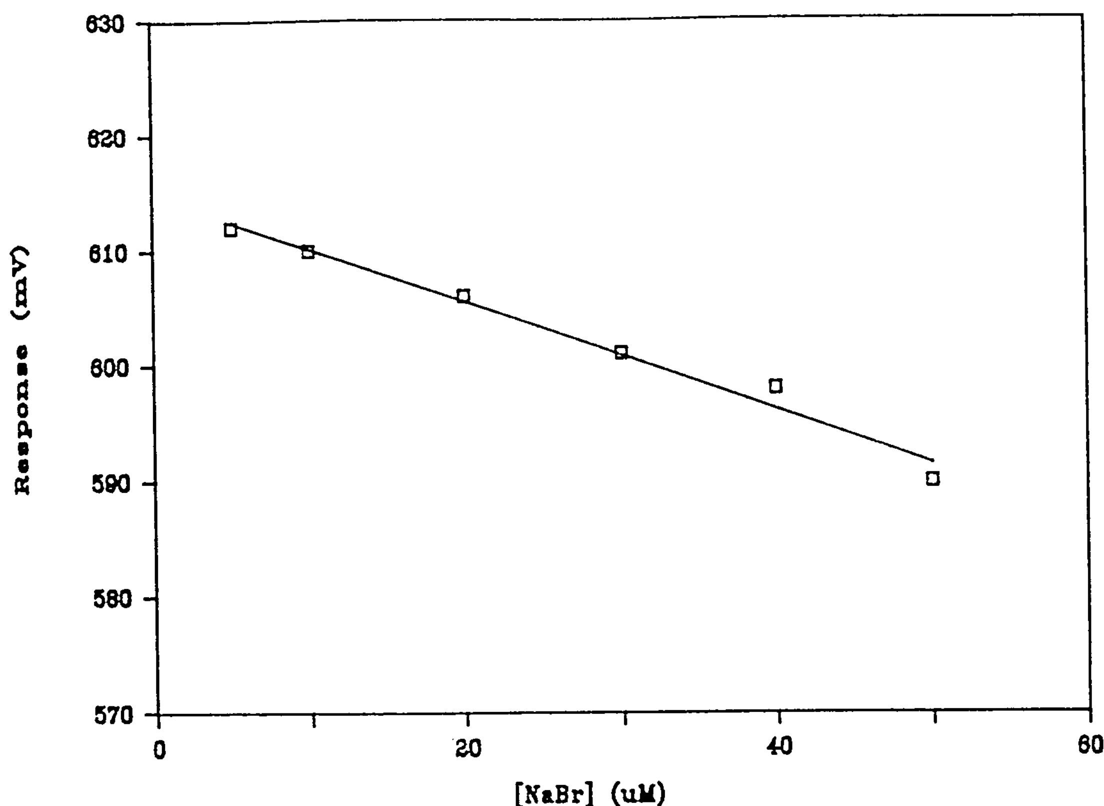


Fig. 3.10: Linearity of the bromide-specific ion-electrode used to determine halothane metabolism (see Section 3.5).
A stock solution of sodium bromide (1 mM initial concentration in H_2O, 5 μM - 50 μM final solution) was diluted into 10 mM Tris.HCl, pH 8.2, (total volume = 980 μl) and 0.1 M sodium nitrate (20 μl) added 1 min later. The voltage (mV) was then measured using the bromide-specific electrode.

3.12 Standard Curves for GSH Assays.

Two GSH assays were used. The first GSH concentrations in the 0 - 1 mM range and the second for GSH concentrations up to 1.4 μM. The first assay was linear at concentrations up to 1 mM GSH (Fig. 3.11).
The alternative assay for micromolar GSH concentrations (used to determine possible GSH conjugation with halothane and DBE) was linear at concentrations of less than 1.4 μM (Fig. 3.12).

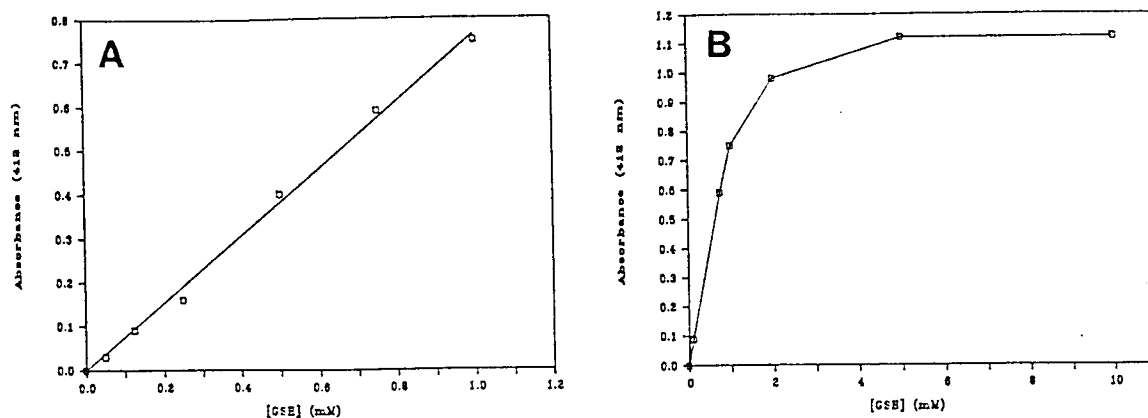


Fig. 3.11: Concentration dependence of the GSH assay at (A) 0 - 1.0 mM and (B) 0 - 10.0 mM GSH.

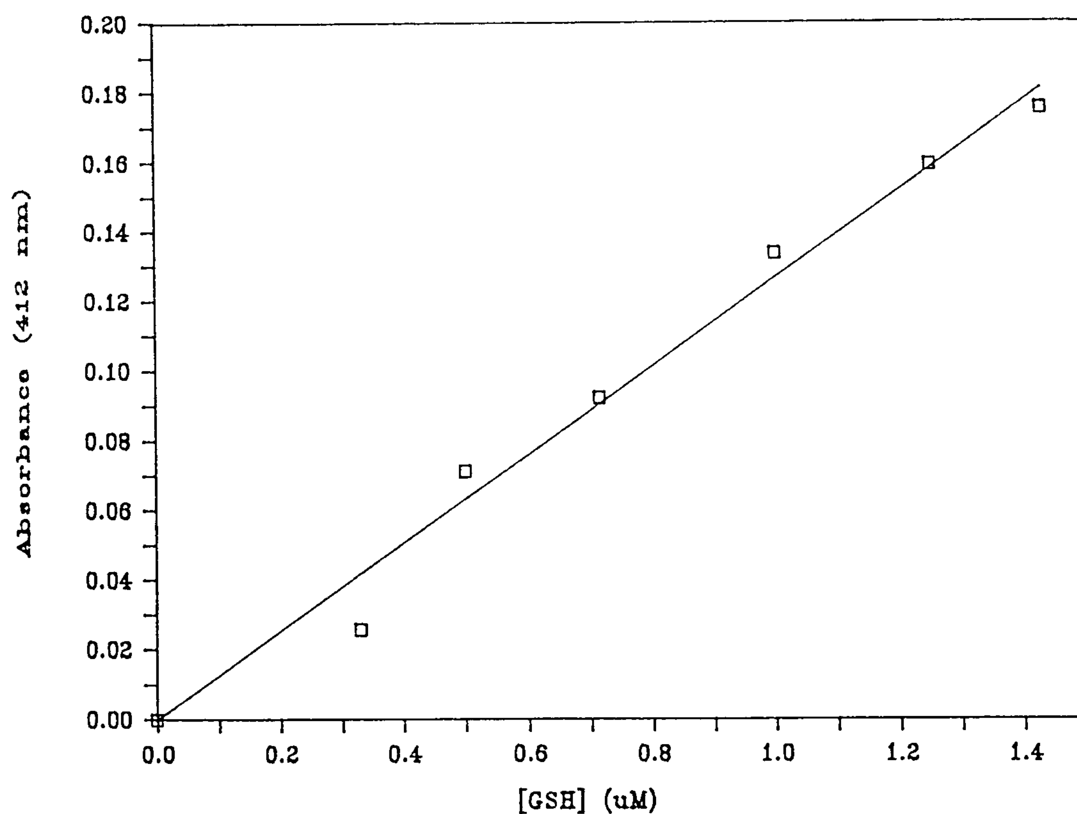


Fig. 3.12: Concentration-dependence of the assay used to determine micromolar concentrations of GSH.

The Reversible Inhibition and Activation of Rat Hepatic GST by 1,2-Dibromoethane in vitro.

The ability of 1,2-dibromoethane to reversibly affect rat hepatic GST activity was measured at various concentrations of both substrates (CDNB & GSH) in a modification of the standard assay. Phosphate buffer containing DBE and CDNB was equilibrated at 25 °C in cuvettes before the addition of GSH and initiation of the assay immediately afterwards with enzyme. This modification was required in view of the low solubility of DBE, which necessitated vigorous vortexing into buffer before addition of the enzyme.

Rat cytosolic GST activity was inhibited at low and activated at high CDNB concentrations (Fig. 4.1). Double reciprocal plots of the data intersected in the first quadrant, indicating mixed-type inhibition/activation with respect to CDNB. The mixture of GST isoenzymes was apparently uncompetitively activated with respect to GSH (Fig. 4.1). An identical pattern of inhibition/activation was obtained with GST 3-3 and 4-4 (Fig. 4.2 & Fig. 4.3).

GST isoenzymes 1-1 and 1-2 were either slightly inhibited or not significantly affected by DBE (Fig. 4.3).

Dilution of EDB, in the presence of cytosolic GST, from 10.4 mM to 5.2 mM and 3.5 mM, by the addition of buffer immediately before assay, decreased enzyme activation from 115 ± 5% to 103 ± 8% and 97 ± 7% (relevant to controls containing ethanol), clearly demonstrating the reversibility of the activation of GST s by DBE.

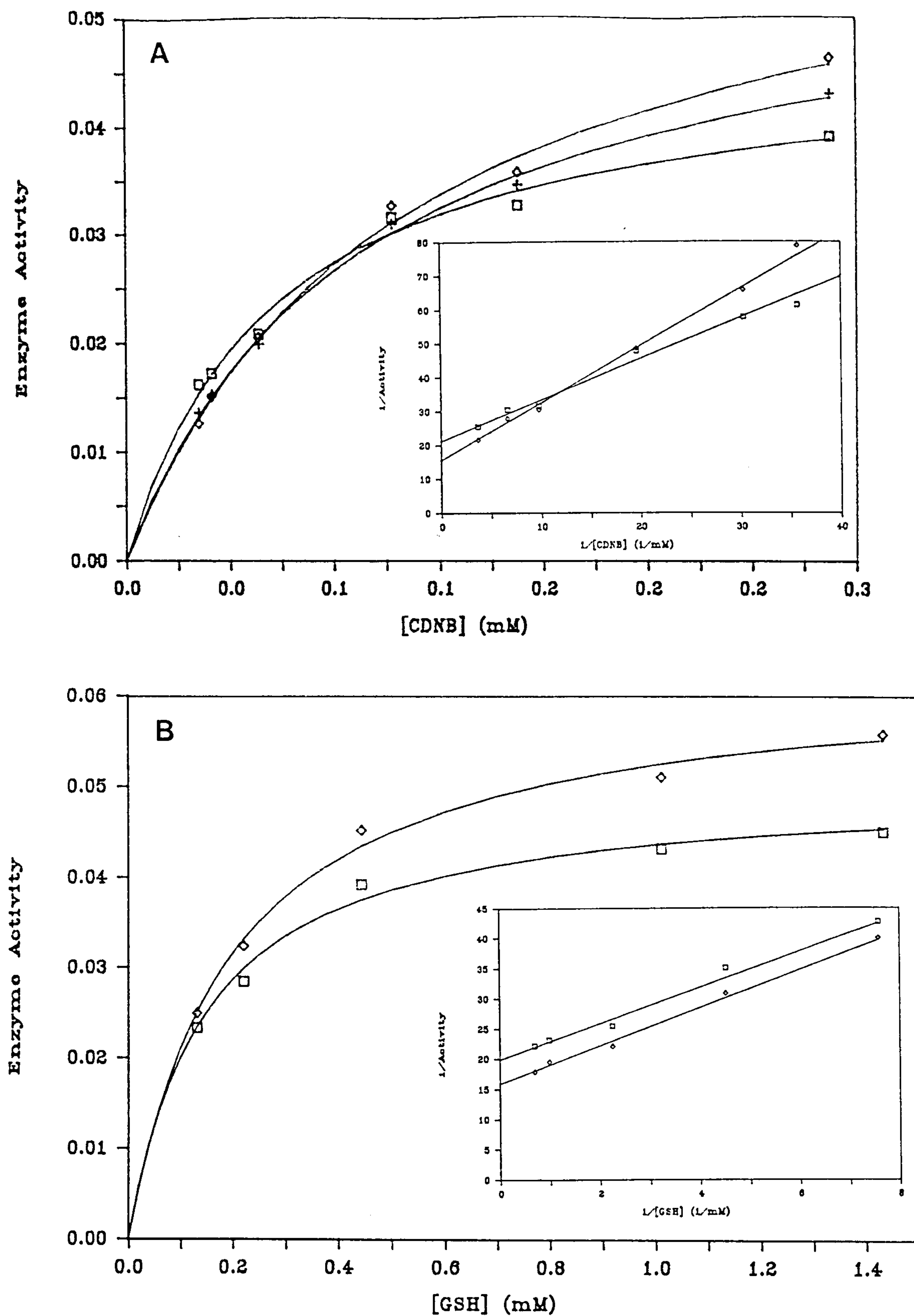


Fig. 4.1: The reversible inhibition and activation of rat cytosolic GST by DBE as a function of CDNB (A) and GSH (B) concentrations. Ethanol (□); 5.85 mM DBE (+); 10.4 mM DBE (◇).
Insets show the relative double-reciprocal plots. Activity is shown as $\Delta A_{340\ nm}$/min. Curves were fitted to the Michaelis-Menten equation by non-linear regression.

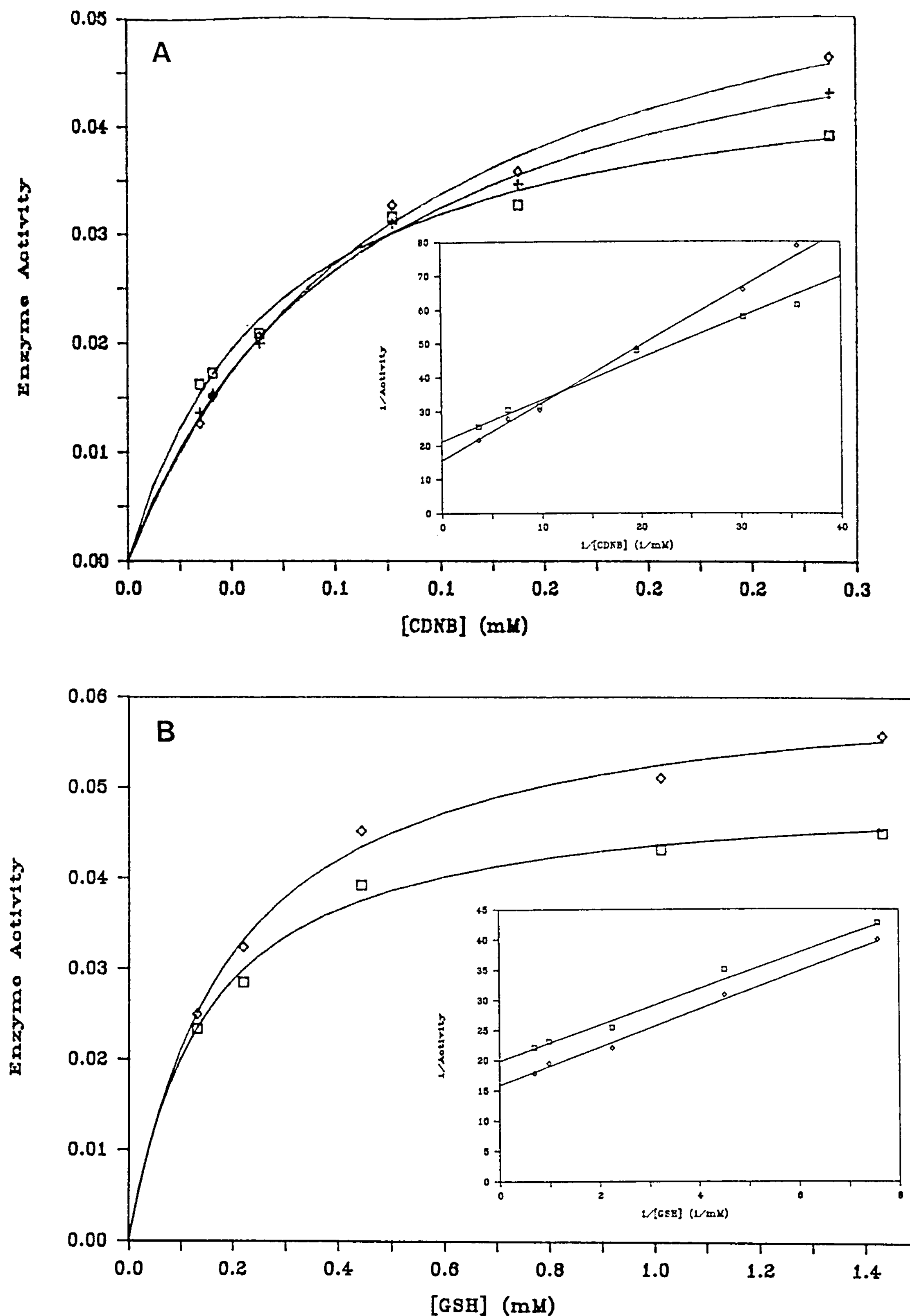


Fig. 4.1: The reversible inhibition and activation of rat cytosolic GST by DBE as a function of CDNB (A) and GSH (B) concentrations. Ethanol (□); 5.85 mM DBE (+); 10.4 mM DBE (◇).
Insets show the relative double-reciprocal plots. Activity is shown as $\Delta A_{340\ nm}$/min. Curves were fitted to the Michaelis-Menten equation by non-linear regression.

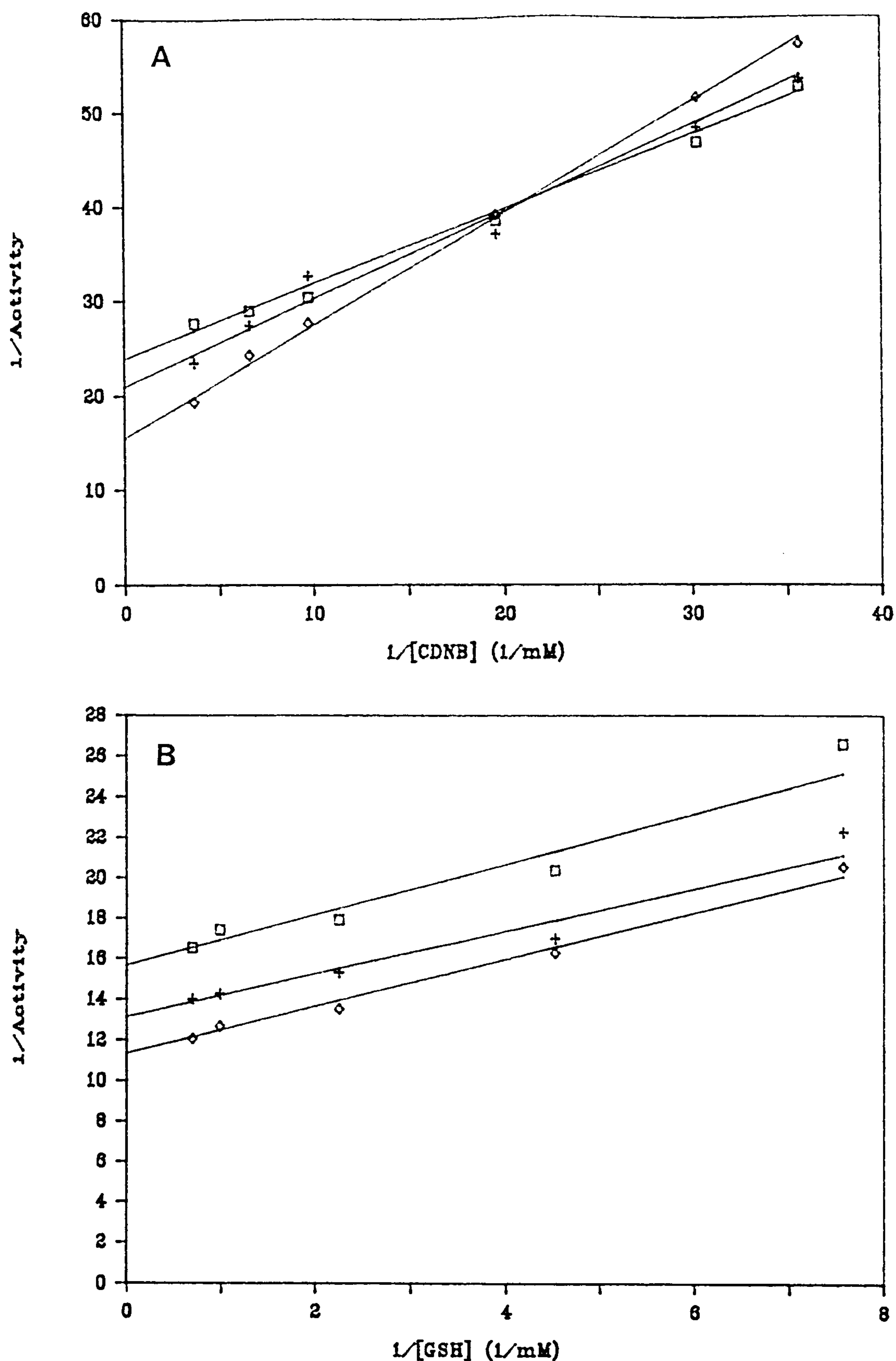


Fig. 4.2: Double-reciprocal plots of reversible inhibition and activation of GST 3-3 by DBE as a function of CDNB (A) and GSH (B) concentrations. Ethanol (□); 5.85 mM DBE (+); 10.4 mM DBE (◇).
Insets display the relative double-reciprocal plots. Activity is shown as $\Delta A_{340\ nm}$/min. Curves were fitted to the Michaelis-Menten equation by non-linear regression.

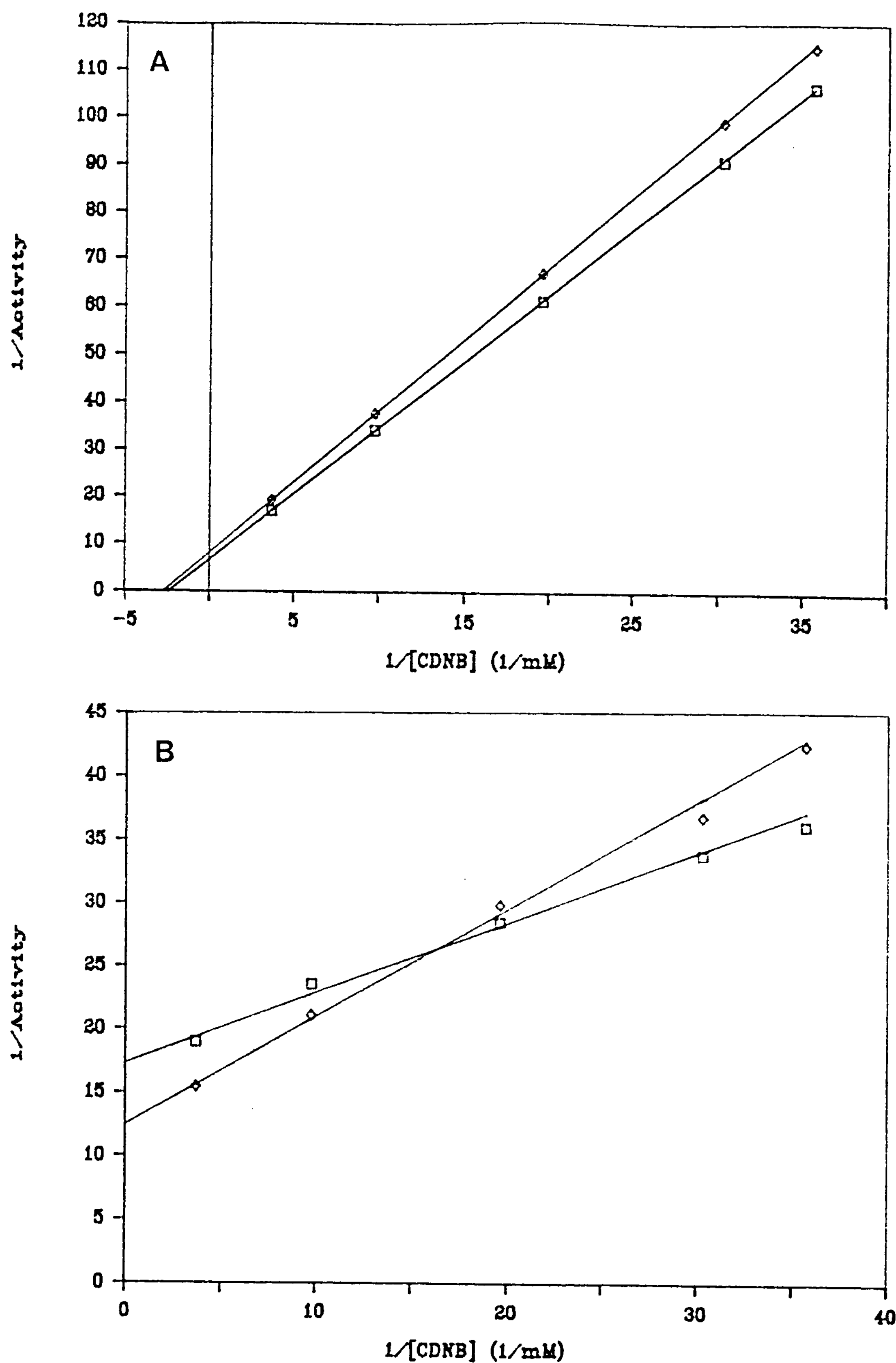


Fig. 4.3: Double-reciprocal plots of reversible inhibition and activation of isoenzymes 1-2 (A) and 4-4 (B) by DBE as a function of CDNB concentration. Ethanol (□); 5.85 mM DBE (+); 10.4 mM DBE (◇).
Activity is shown as $\Delta A_{340\ nm}$/min. Curves were fitted to the Michaelis-Menten equation by non-linear regression.

Bilirubin: Inhibition of GST 1-2

5.1 Time-Dependent Inhibition of GST 1-2 by Bilirubin.

The time-dependent inhibition of GST 1-2 by bilirubin exhibited pseudo-first order kinetics with observed rate constants of 0.0214 ± 0.0005 s^{-1} and 0.040 ± 0.008 s^{-1} at 4 and 8 μM bilirubin, respectively (Fig. 5.1). These values correspond to calculated second-order rate constants of 5.3 ± 0.1 x 10^3 and 5.0 ± 1.0 x 10^3 $M^{-1}.s^{-1}$, respectively.

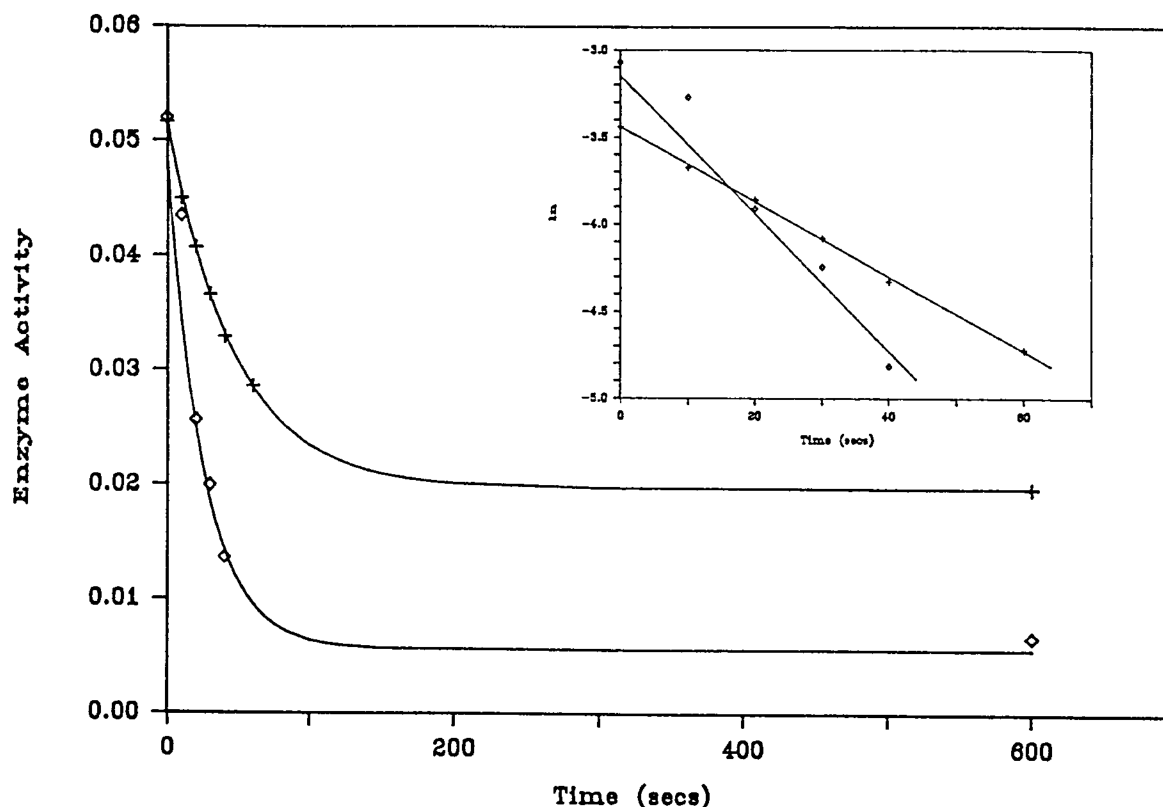


Fig. 5.1: Time-dependent inhibition of GST 1-2 in the presence of 4 μM (+) and 8 μM (◇) bilirubin. GST 1-2 (4 nM) was incubated with bilirubin in 20 mM potassium phosphate containing 0.1 M NaCl, pH 6.5, at 25 °C. Enzyme rates are shown as $\Delta A_{340\ nm}$/min and were corrected for loss of activity in the absence of bilirubin.
Curves were fitted to a three parameter decay equation:

$$A_t = A_\infty - (A_0 - A_\infty)e^{-k_1 t}.$$

Insert displays a plot of $\ln(A_t - A_\infty)$ vs time.

The extent of inhibition of GST 1-2 was dependent on bilirubin concentration with half-maximal inhibition at approximately 4 μM bilirubin (Table 5.1).

Table 5.1: Concentration dependence of bilirubin inhibition of GST 1-2.

Bilirubin (μM)	Activity (%)
0	88.2 ± 0.3
0.5	84.1 ± 2.4
1.0	73.5 ± 2.4[a]
2.0	62.3 ± 0.4[a]
4.0	43.5 ± 6.7[a]
8.0	6.2 ± 2.3[a]

GST 1-2 (4 nM) activity was measured after incubation with bilirubin in 20 mM potassium phosphate and 100 mM NaCl, pH 6.5, for 20 min at 25 °C. Activity (%) is relative to identical zero time samples in the absence of bilirubin.
[a] Differs from samples in the absence of bilirubin, $P < 0.001$.

5.2 Reversal of Bilirubin Inhibition.

Inhibition of GST 1-2 by bilirubin was reversed by dilution: The enzyme (4 nM) was incubated with bilirubin (8 μM) at room temperature for 3 min, diluted 10-fold into buffer containing bilirubin (8 μM) or NaOH (0 - 50 μl), incubated for a further 5 min and assayed for enzyme activity. Dilution of enzyme 10-fold into 8 μM bilirubin resulted in a complete loss of enzyme activity, while 10-fold dilution of both the enzyme and bilirubin concentrations (*viz* 0.8 μM bilirubin final concentration), caused only a 26% decrease in activity. Percentage activities are relative to enzyme samples treated exactly as above, but in the absence of 8 μM bilirubin in the first step.

Bilirubin inhibition was pH-dependent and reversed by increasing the pH. Incubation of GST 1-2 with 8 μM bilirubin for 3 min reduced enzyme activity to 0% and 55 ± 1% at pH 6.0 and 7.4, respectively, compared to identical zero time samples. Following incubation of the enzyme at pH 6.0 with 8 μM bilirubin for 3 min, the pH was adjusted to 7.4 and enzyme activity measured at 0, 2 and 4 min after the pH increase. Under these conditions enzyme activity returned to 69 ± 2%, 92 ± 18% and 118 ± 33% relative to enzyme incubated at pH 7.4. in the absence of bilirubin.

5.3 Effect of Albumin on GST 1-2 Inhibition.

Incubation of GST 1-2 in buffer in the presence or absence of CDNB resulted in approximately a 10% loss in activity, with albumin appearing to minimize the loss of activity (Table 5.2). Bilirubin significantly inhibited the enzyme. Pre-mixing bilirubin with albumin, before the addition of GST, effectively prevented or greatly reduced inhibition by bilirubin. In contrast, the enzyme was more strikingly inhibited when GST and albumin were mixed before the addition of bilirubin, although the extent of inhibition was not as large as in the absence of albumin.

Table 5.2: Effect of bilirubin and albumin on GST 1-2 activity.

Additions	Activity (%)
GST	89.0 ± 0.3
CDNB + GST	88.4 ± 1.3
HSA + GST	98.4 ± 1.8
(CDNB + HSA) + GST	100.4 ± 0.3
(8 μM BR + CDNB) + GST	0.0[a]
(8 μM BR + CDNB + HSA) + GST	93.3 ± 0.1[b]
(CDNB + HSA + GST) + 8 μM BR	75.2 ± 2.0[a,b,c]
(35 μM BR + CDNB + HSA) + GST	69.6 ± 1.9[a,b]
(CDNB + HSA + GST) + 35 μM BR	26.5 ± 1.8[a,b,c]

GST 1-2 (4 nM) activity was measured after incubation for 15 min at 25 °C in the presence or absence of bilirubin (BR), albumin (HSA; 2 μM) and CDNB (1 mM). Components were added to the incubations in the order indicated, with components in parenthesis well mixed prior to subsequent additions. The enzyme assay was initiated with GSH (2.5 mM). Activity (%) is relative to identical zero time samples.

[a] Differs significantly from activity for GST or CDNB and GST, $P < 0.001$
[b] Differs significantly from activity for (BR + CDNB) and GST, $P < 0.001$
[c] Differs significantly from activity for (BR + HSA) and GST, $P < 0.001$

5.4 Bilirubin Binding by Proteins.

The binding of bilirubin by various proteins was assessed by a number of methods, of these gel filtration on Sephadex G-25 proved to be the method of choice (see below for other methods examined). Protein and protein-bound bilirubin was eluted from Sephadex columns with buffer, whereas free bilirubin, which bound to the Sephadex, was recovered by washing the columns with 10 mM NaOH. In the absence of bilirubin, protein recovery in the buffer wash was between 84% and 108% (assessed spectrophotometrically at 280 nm). Bilirubin recovery, in the

absence of protein, was between 93% and 136% in the NaOH wash (determined at 468 nm).

The ability of albumin, aldolase, hemoglobin and ribonuclease to bind bilirubin was determined by Sephadex chromatography (Fig 5.2). Protein-bound bilirubin was assumed to not bind to the Sephadex resin (see above) whereas free bilirubin bound to the resin and was eluted separately with 10 mM NaOH. Free bilirubin concentrations (*ie* that not bound to protein) decreased, and protein-bound bilirubin levels increased, with rising concentrations of albumin, hemoglobin and aldolase. These proteins decreased free bilirubin concentrations by greater than 75% at protein concentrations greater than 0.05 μM (molar ratios of [bound bilirubin]/[protein] of up to 100 at approximately 0.04 μM protein) (Fig. 5.2). Under identical conditions, ribonuclease ($\leq$ 16 μM) appeared to bind less than 15% of the bilirubin (Fig. 5.2).

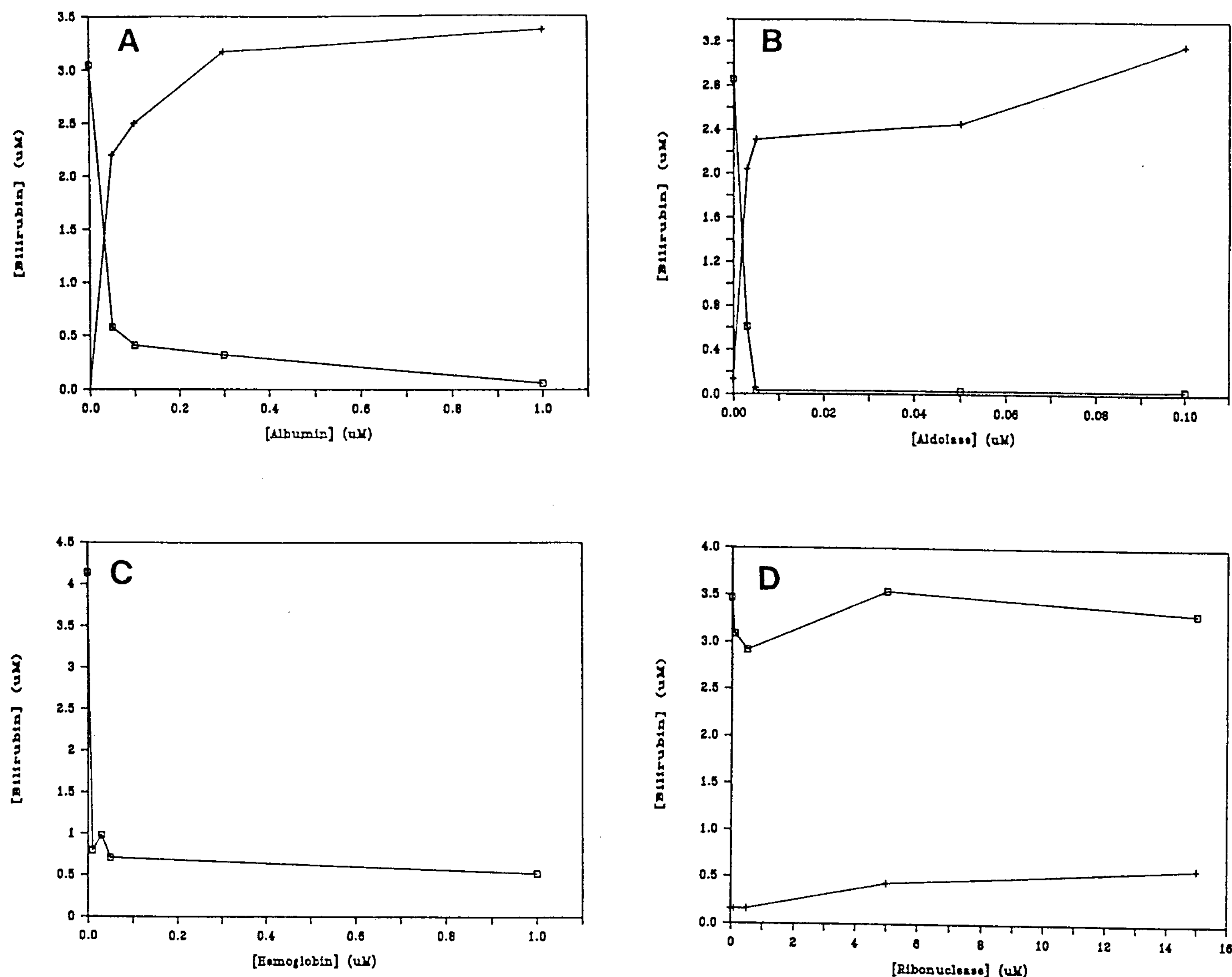


Fig. 5.2: Concentrations of free (□) and protein-bound (+) bilirubin in eluates from Sephadex G-25 columns as a function of protein concentration: (A) Albumin; (B) Aldolase; (C) Hemoglobin; (D) Ribonuclease. Proteins were mixed with bilirubin (4 μM) before application (1.0 ml) onto Sephadex G-25 columns (1.0 x 0.8 cm); Protein-bound bilirubin was eluted with phosphate buffer (7.0 ml), and free bilirubin was eluted with 10 mM NaOH (10.0 ml).

5.5 Protection against Bilirubin Inhibition by Proteins.

Concentrations of albumin, hemoglobin and aldolase (0.001 - 0.01 μM), which did not significantly reduce concentrations of free bilirubin (Fig. 5.2), had no effect on bilirubin inhibition of GST 1-2 (Table 5.3). In contrast, higher concentrations of these proteins (0.2 - 0.4 μM) prevented inhibition by bilirubin, in direct proportion to their ability to bind free bilirubin (Table 5.3, Fig. 5.2). Ribonuclease, which did not appear to bind bilirubin (Fig. 5.2), also prevented inhibition at a concentration of greater than 0.5 μM, although low concentrations (0.004 μM) were not effective in preventing inhibition (Table 5.3).

Table 5.3: Protection against bilirubin inhibition of GST 1-2 by proteins.

Additions	Activity (%)	Additions	Activity (%)
None	84.1 ± 0.2	None	88.2 ± 3.7
Albumin (0.01 μM)	83.4 ± 2.4	Hemoglobin (0.001 μM)	94.3 ± 3.5
Albumin (0.4 μM)	90.9 ± 1.9	Hemoglobin (0.2 μM)	97.9 ± 3.0
Bilirubin	54.2 ± 0.5[a]	Bilirubin	48.7 ± 0.9[a]
Bilirubin + albumin (0.01 μM)	54.0 ± 0.1[a]	Bilirubin + hemoglobin (0.001 μM)	51.0 ± 5.2[a]
Bilirubin + albumin (0.4 μM)	78.4 ± 0.4[b]	Bilirubin + hemoglobin (0.2 μM)	95.2 ± 1.2[b]
None	98.3 ± 0.4	None	94.3 ± 2.7
Aldolase (0.001 μM)	80.3 ± 5.9	Ribonuclease (0.004 μM)	76.6 ± 2.7
Aldolase (0.3 μM)	93.3 ± 0.4	Ribonuclease (0.5 μM)	91.2 ± 3.1
Bilirubin	53.6 ± 3.7[a]	Bilirubin	55.2 ± 0.7[a]
Bilirubin + aldolase (0.001 μM)	55.8 ± 1.0[a]	Bilirubin + ribonuclease (0.004 μM)	55.2 ± 0.7[a]
Bilirubin + aldolase (0.3 μM)	89.4 ± 0.5[b]	Bilirubin + ribonuclease (0.5 μM)	97.1 ± 1.2[b]

The relevant proteins were mixed with 4 μM bilirubin in 20 mM potassium phosphate/100 mM NaCl, pH 6.5, before addition of GST 1-2 (4 nM) and incubation for 20 min at 25 °C.

[a] Differs significantly from activity with no additions, $P < 0.001$

[b] Differs significantly from activity with bilirubin, $P < 0.001$

5.6 Effect of Different Phosphate Buffers on Inhibition.

Incubation of GST 1-2 with bilirubin (8 μM) in 0.1 M potassium phosphate (pH 6.5) decreased the activity to 31 ± 2% after a 5 min incubation. Incubation in 20 mM phosphate containing 0.1 M NaCl (pH 6.5) decreased activity to 35 ± 4%. The ionic strength therefore did not significantly affect bilirubin inhibition of GST 1-2.

5.7 Alternative Methods used to Measure Free Bilirubin Concentrations.

Although bilirubin has a low solubility in water, it is soluble in basic aqueous solution and in organic solvents (Brodersen, 1980) The accurate determination of protein-bound bilirubin concentrations is therefore not a simple process and various approaches were therefore tested, Sephadex chromatography was found to be the best of these although still not an ideal method. The following methods were also attempted:

5.7.1 The Use of Ultrafiltration Membranes to Separate Albumin and Bilirubin.

Albumin (8 μM) and bilirubin (35 μM) were separately added to an Amicon PM-10 ultrafiltration membrane (MW cutoff = 20 kdaltons), and the relevant concentrations measured spectrophotometrically in the filtrate. Albumin (MW ≈ 60 kdaltons) was virtually completely retained in the concentrate (≥ 95%). However, contrary to expectations, bilirubin (MW = 584.7 daltons) was also retained (≥ 97%). It appeared that bilirubin was bound to the membrane (as determined by the membrane being a dark red color), probably as a result of its high hydrophobicity, and could only be removed by washing the membrane with 10 mM NaOH.

5.7.2 Use of Cation-Exchangers to Separate Protein and Bilirubin.

Attempts were made to separate proteins from bilirubin by ion-exchange chromatography (Table 5.4). Only one resin, Amberlite CG50, bound ribonuclease significantly (75% binding), but this resin also bound bilirubin (60%). Attempts to saturate ion-exchange resins in columns with bilirubin were not successful as bilirubin only bound to the top of the resin. The resin was also not be saturated by mixing with bilirubin in a beaker.

5.7.3 Precipitation of Proteins with Organic Solvents and TCA.

Neither acetone (50% final volume) nor TCA (70% final concentration) significantly precipitated albumin, ribonuclease or hemoglobin (8 μM). Ammonium sulfate (60 - 70%) did not precipitate any of the proteins, but did precipitate albumin at 80% concentration. The latter process required filtration as the solution appeared to be saturated and the ammonium sulfate was not completely dissolved. Bilirubin was retained by the filter paper and this method could therefore not be used.

Table 5.4: Binding of bilirubin and proteins to ion-exchange resins.

		% absorbance of supernatant Resin			
		Dowex AG50	Amberlite CG120	Amberlite CG50	CM-cellulose
Ligand	(μM)				
Bilirubin	(35)	55	61	40	15
Hemoglobin	(0.1)	100	583	122	N.D.[a]
Ribonuclease	(5.0)	111	145	25	118

Samples were mixed with the relevant ion-exchange resin (1 g) and the amount of ligand bound to the resin determined by absorbance measurements of the supernatant. Binding was allowed to take place for 5 min with intermittent stirring. Bilirubin concentration was measured at 468 nm and protein at 280 nm (bilirubin and protein were analyzed separately).
The cation-exchangers were equilibrated with 0.1 M potassium phosphate (pH 6.5) before use. All the resins were effective at pH 6.5, according to the manufacturers instructions.
[a] N.D.: Not determined.

5.7.4 Bilirubin Extraction by Organic Solvents.

Bilirubin (35 μM) in 0.1 M phosphate, pH 6.5, was extracted with equal volumes of organic solvents (chloroform, benzene, toluene, carbon tetrachloride). The solvents extracted more than 95% bilirubin from the buffer.
Extraction of protein solutions with these solvents caused the formation of a protein interface between the aqueous and organic phases, although these were less intense with chloroform and carbon tetrachloride. Extraction with carbon tetrachloride resulted in the loss of 15% - 20% of the protein sample from the aqueous phase, while less than 10% protein was apparently lost with chloroform, although hemoglobin could not be assayed by this method since the aqueous phase was cloudy. Chloroform extracted all of the bilirubin from samples containing protein, and therefore was not suitable for assessing bilirubin binding by proteins.

5.7.5 Centrifugation of Bilirubin and Proteins.

Hemoglobin, albumin and bilirubin were centrifuged at 280 000g for eight hours to pellet the protein and separate it from bilirubin in the supernatant. However, although hemoglobin (0.008 - 8.0 μM) and albumin (0.0125 - 2.0 μM) did precipitate, bilirubin (2 - 32 μM) appeared to aggregate and bind to the sides of the centrifugation tubes.

5.7.6 Precipitation with Sulfuric Acid and Sodium Tungstate.

Proteins can be precipitated by the addition of sulfuric acid (40 mM) and sodium tungstate (12 mM) and subsequent centrifugation at 2000 rpm for 5 min (Costa & Ivanetich, 1984). Proteins were precipitated by this method, but bilirubin was also significantly precipitated (data not shown).

Kinetic Mechanism of Erythrocyte GST p (rho).

6.1 Kinetic Studies of GST ρ.

The kinetic mechanism of GST ρ was investigated by measuring initial conjugation rates at eight concentrations of the substrates CDNB and GSH. The range of concentrations were 0.25 - 2.00 mM for CDNB and 0.05 - 1.00 mM for GSH. Thus, a total of 64 different GSH/CDNB combinations (8 x 8 matrix) were used and all rate determinations were performed in one experiment (experiments were repeated).

The data were fitted to the Michaelis-Menten equation by non-linear regression. Double reciprocal plots of the data showed that the fitted lines intersected below the x-axis (Fig. 6.1), indicating a random rapid equilibrium type mechanism with $\alpha > 1$ (Segel, 1975).

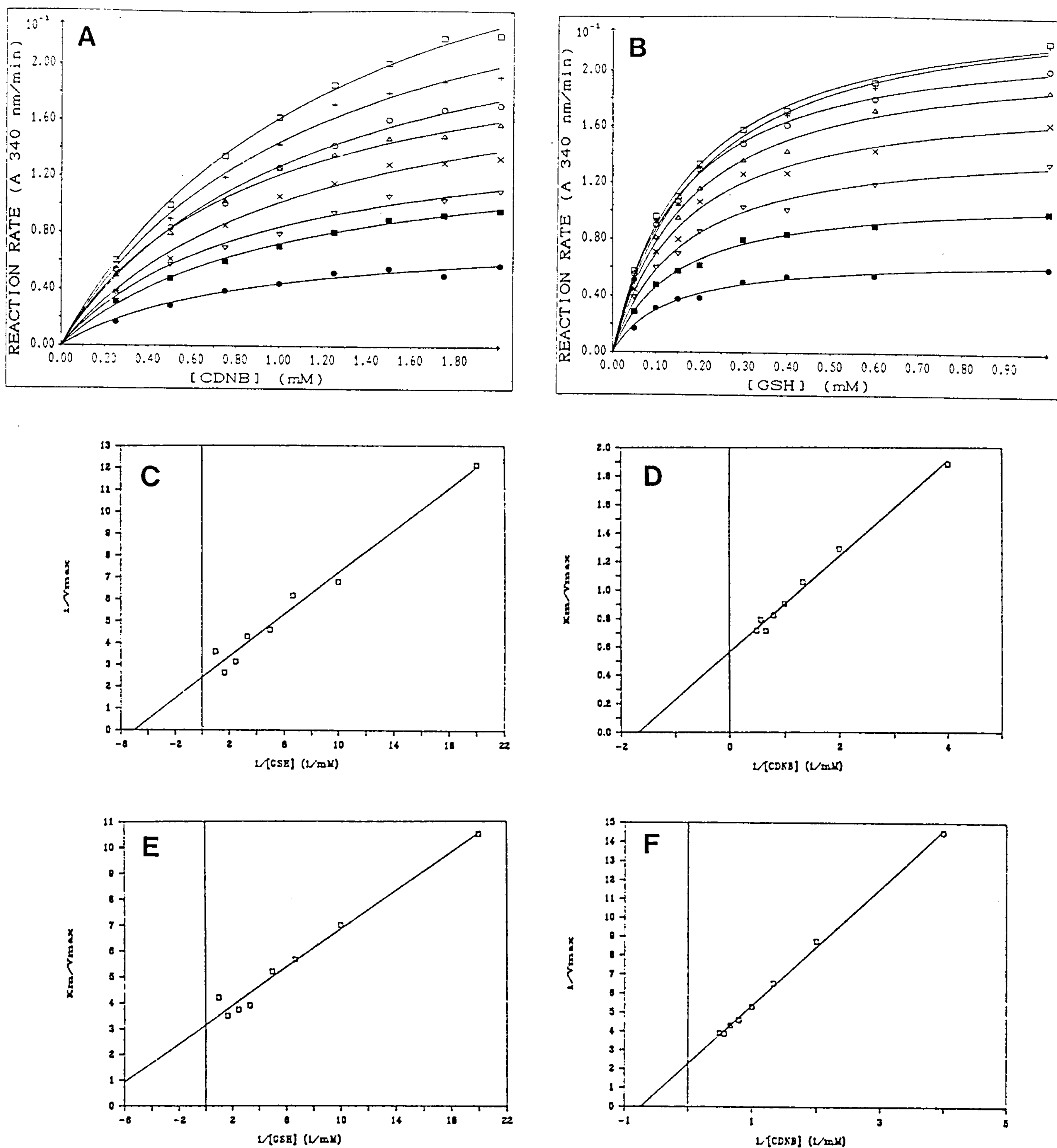


Fig 6.1: Plots of initial rate data with curves fitted to the Michaelis-Menten equation (A, B) and replots of the obtained kinetic parameters (C, D, E, F). The GSH concentrations used in plot A were 0.05 mM (●), 0.10 mM (■), 0.15 mM (▽), 0.20 mM (×), 0.30 mM (△), 0.40 mM (○), 0.60 mM (+), and 1.00 mM (□).
The CDNB concentrations used in plot B were 0.25 mM (●), 0.50 mM (■), 0.75 mM (▽), 1.00 mM (×), 1.25 mM (△), 1.50 mM (○), 1.75 mM (+), and 2.00 mM (□).

Subsequently the data were fitted to various kinetic mechanisms (Table 6.1) using non-linear regression to obtain kinetic parameters and appropriate statistical limits (Table 6.2). Discrimination between kinetic models was made using the following criteria: (a) parameter values, (b) convergence, (c) randomness of distribution of residuals, and (d) residual sum of squares (Mannervik, 1981).
Ordered bireactant and ping pong mechanisms were eliminated because of lack of convergence, large residual sums squared (Table 6.2) or non-random distribution of residuals (not shown).
A random BiBi steady state mechanism provided the lowest residual sum of squares with the analyzed data (Table 6.2). However, two parameters were either equal to zero (V_2, K_7), and a third did not differ significantly from zero (K_4) (Table 6.2); Furthermore, the residuals at lower CDNB concentrations were not randomly distributed (Fig. 6.2). It was therefore therefore hypothesized that the random steady state mechanism could be overfitting the data and experiments were performed at higher GSH concentrations (0.25 - 8 mM) to assess possible adherence to simple Michaelis-Menten kinetics at high substrate concentrations. The results of these experiments were consistent with Michaelis-Menten kinetics (Fig. 6.3), and the random steady state mechanism was accordingly rejected in favor of the random rapid equilibrium mechanism.

Replots of the data (Fig. 6.1) were consistent with a random rapid equilibrium mechanism, whereas non-linear regression analysis (Table 6.2) indicated either a random rapid equilibrium or an ordered steady state mechanism. In the absence of product the steady-state rate equations for these mechanisms are identical (Segel, 1975). Product inhibition studies were thus used to differentiate between them.

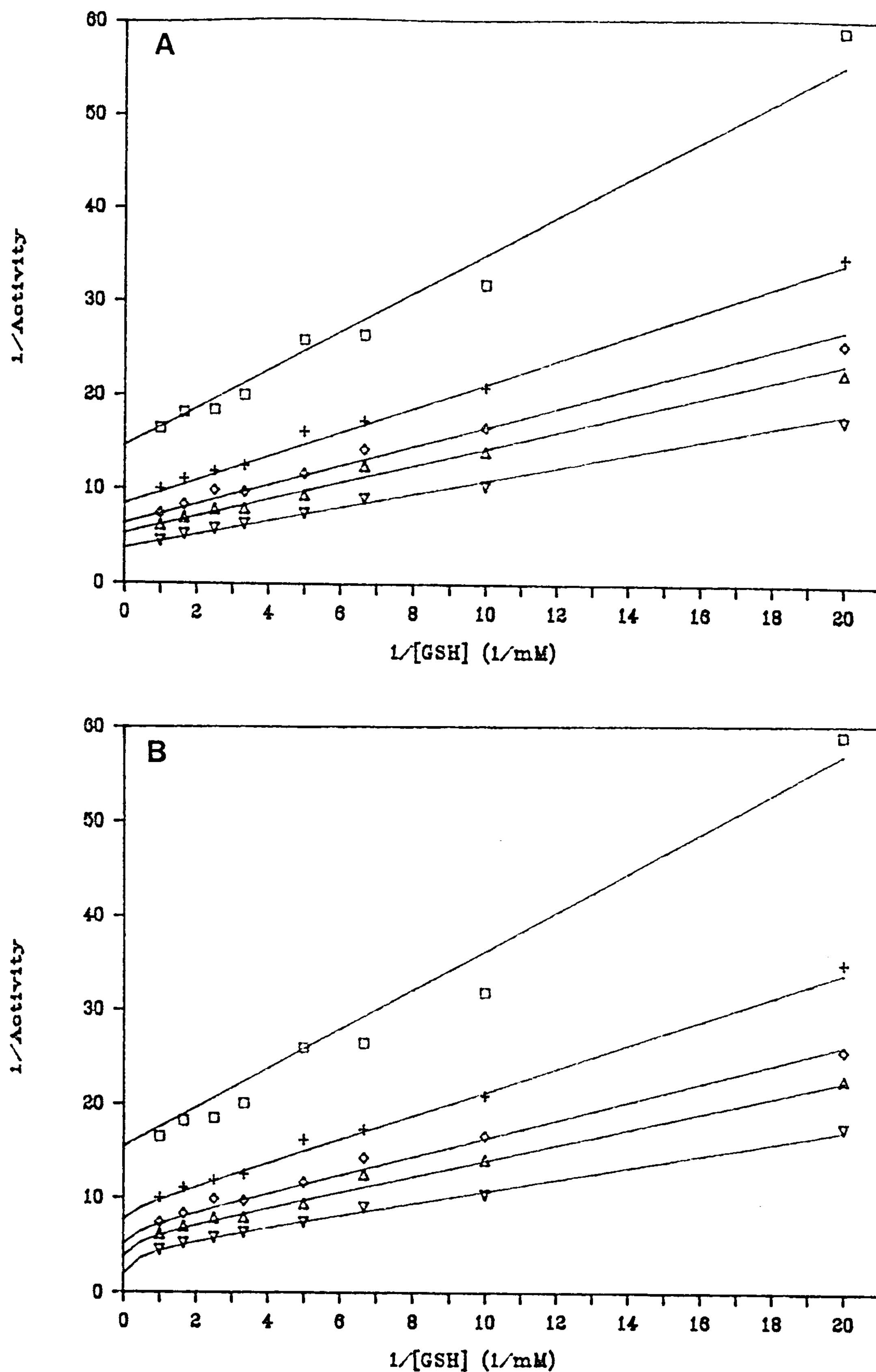


Fig. 6.2: Double-reciprocal plots of initial rates as a function of GSH concentrations in the absence of product. The CDNB concentrations were 0.25 mM (□), 0.50 mM (+), 0.75 mM (◇), 1.00 mM (△), and 2.00 mM (▽). The data were fitted to (A) a random rapid equilibrium mechanism and (B) a random steady state mechanism. Activity is shown as $\Delta A_{340\ nm}$/min.

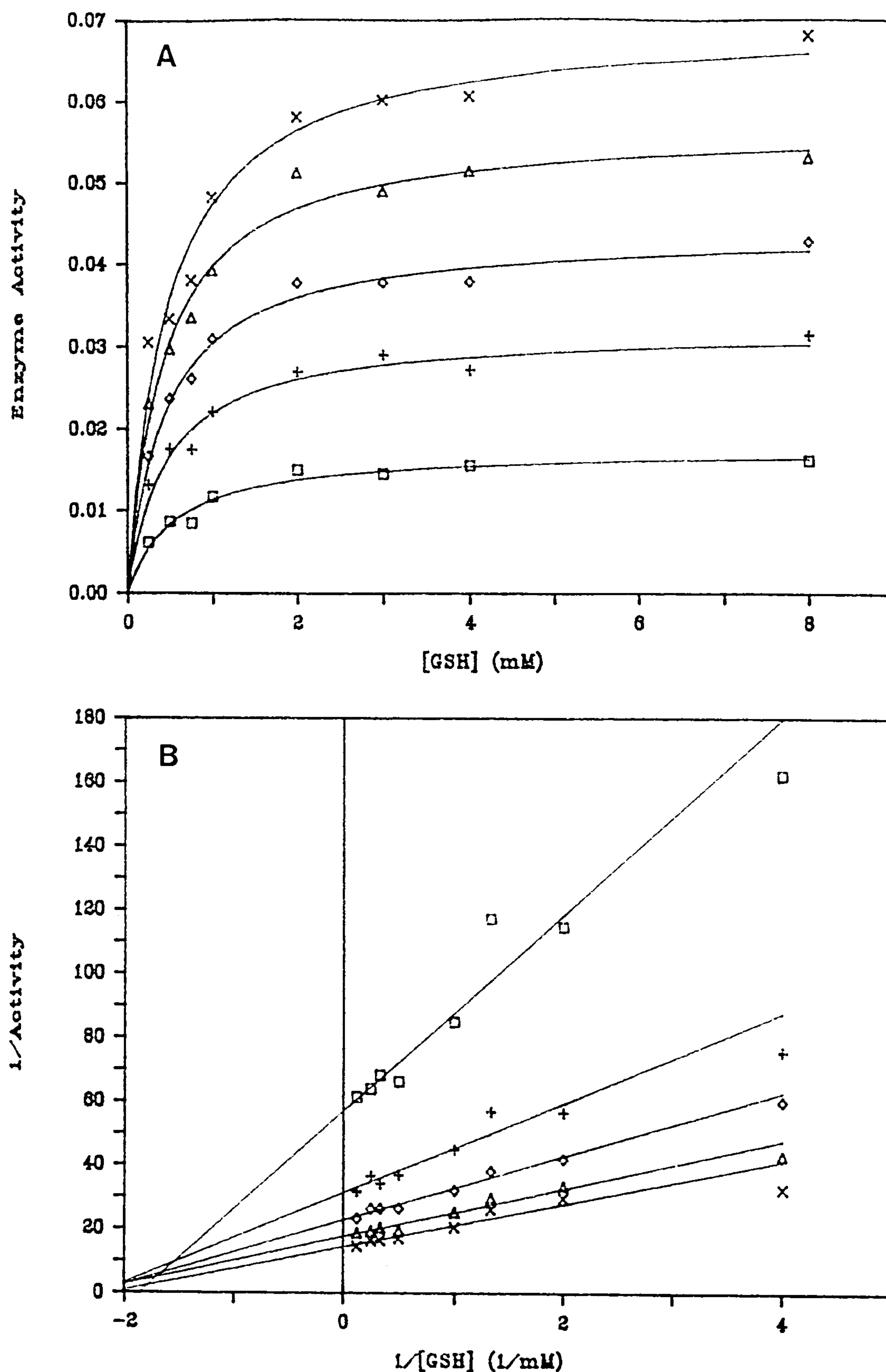


Fig. 6.3: Initial rates at different GSH and constant CDNB concentrations. CDNB concentrations were 0.25 mM (□), 0.50 mM (+), 0.75 mM (◇), 1.00 mM (△), and 1.50 mM (×).
(A) Substrate/velocity and (B) double-reciprocal plots. The curves show the relevant data fitted to the Michaelis-Menten equation. Activity is shown as $\Delta A_{340\ nm}$/min.

Table 6.1: Rate equations for bireactant kinetic mechanisms.

Random rapid equilibrium mechanism:

$$\frac{v}{V_{max}} = \frac{[A]\,[B]}{\alpha K_A K_B + \alpha K_A[B] + \alpha K_B[A] + [A]\,[B]}$$

Ordered rapid equilibrium mechanism:
(A binds before B)

$$\frac{v}{V_{max}} = \frac{[A]\,[B]}{K_A K_B + K_B[A] + [A]\,[B]}$$

Ordered steady state mechanism:
(A binds before B)

$$\frac{v}{V_{max}} = \frac{[A]\,[B]}{K_{ia}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A]\,[B]}$$

Ping pong mechanism:

$$\frac{v}{V_{max}} = \frac{[A]\,[B]}{K_{mA}[B] + K_{mB}[A] + [A]\,[B]}$$

Random steady state mechanism:[a]

$$v = \frac{V_1AB + V_2A^2B + V_3AB^2}{K_1 + K_2A + K_3B + K_4A^2 + AB + K_5B^2 + K_6A^2B + K_7AB^2}$$

In the above equations v refers to the initial rate of the conjugation reaction, with V_{max} the maximum attainanble rate. A and B are arbitrary references to the substrates CDNB and GSH, respectively. Michaelis constants (K) are combinations of microscopic rate constants (Segel, 1975).

[a] $V_{(1-3)}$ are constants related to E_T; $K_{(1-7)}$ are combinations of rate constants (Mannervik & Askelöf, 1975).

Table 6.2: Kinetic parameters obtained by fitting the initial rate data to different kinetic mechanisms.

Random Rapid Equilibrium Mechanism

Kinetic parameters:

V_{max} = 0.45 ± 0.02 α = 2.0 ± 0.4
K_{GSH} = 0.12 ± 0.02 K_{CDNB} = 0.70 ± 0.11

Statistical parameters:

RSS[a] = 0.0052 R^2 = 0.99 Variance = 0.0026
DF[a] = 188 MSE[a] = 0.000028 DF[a] = 185

Random Steady State Mechanism

Kinetic parameters:

V_1 = 0.45 ± 0.12 V_2 = 0 V_3 = 0.18 ± 0.07
K_1 = 0.20 ± 0.03 K_2 = 0.20 ± 0.11
K_3 = 1.72 ± 0.59 K_4 = 0.0.003 ± 0.028[b]
K_5 = 0.70 ± 0.39 K_6 = 0.22 ± 0.14 K_7 = 0

Statistical parameters:

RSS = 0.0041 R^2 = 0.99 Variance = 0.0026
DF = 188 MSE = 0.000023 DF = 181

Ordered Rapid Equilibrium Mechanism[c]

Kinetic parameters:

V_{max} = 0.30 ± 0.01 K_{GSH} = 0.63 ± 0.05
K_{CDNB} = 0.51 ± 0.05

Statistical parameters:

RSS = 0.013 R^2 = 0.97 Variance = 0.0026
DF = 188 MSE = 0.000071 DF = 186

Ordered Steady State Mechanism[c,d]

Kinetic parameters:

V_{max} = 0.45 ± 0.02 K_{GSH} = 0.24 ± 0.02
K_{CDNB} = 1.41 ± 0.10 $K_{i(GSH)}$ = 0.12 ± 0.02

Statistical parameters:

RSS = 0.0052 R^2 = 0.99 Variance = 0.0026
DF = 188 MSE = 0.000028 DF = 185

Ping Pong Mechanism

Kinetic parameters:

V_{max} = 0.66 ± 0.03 K_{GSH} = 0.53 ± 0.03
K_{CDNB} = 2.70 ± 0.18

Statistical parameters:

RSS = 0.0085 R^2 = 0.98 Variance = 0.0026
DF = 188 MSE = 0.000046 DF = 186

Initial rates (189 values) were fitted to the kinetic mechanisms by non-linear regression using the BMDP statistical analysis program. Data were weighted according to the inverse of the variance of the calculated rates obtained by linear regression.

Enzyme rates are shown as $\Delta A_{340\ nm}$/min; Michaelis constants (K) are given in units of concentrations (mM).

[a] RSS = Residual sums squared; MSE = Estimated mean square error; DF = Degrees of freedom for the variance and estimated mean squared error, respectively.

[b] This parameter is not significantly different from zero.

[c] Ordered addition of substrates refers to GSH as the first substrate.

[d] In the case of CDNB binding first, the value of $K_{i(CDNB)}$ is calculated to be 0.70 ± 0.10 mM.

6.2 Product Inhibition Studies.

Inhibition of GST ρ by S-(2,4-dinitrophenyl)GSH, the product of the reaction between CDNB and GSH, was measured at a number of concentrations of the first substrate, with the second substrate fixed at non-saturating concentrations and *vise versa* (Segel, 1975). The conjugate demonstrated competitive and mixed-type inhibition with respect to GSH and CDNB (Fig. 6.4) indicating either an ordered bibi or a random rapid equilibrium mechanism with an enzyme-CDNB-conjugate dead-end inhibition complex. Replots of the inhibition data (Fig. 6.5) showed that $K_{i(slope)}$ was dependent on the concentration of CDNB, indicating a random system (Segel, 1975).

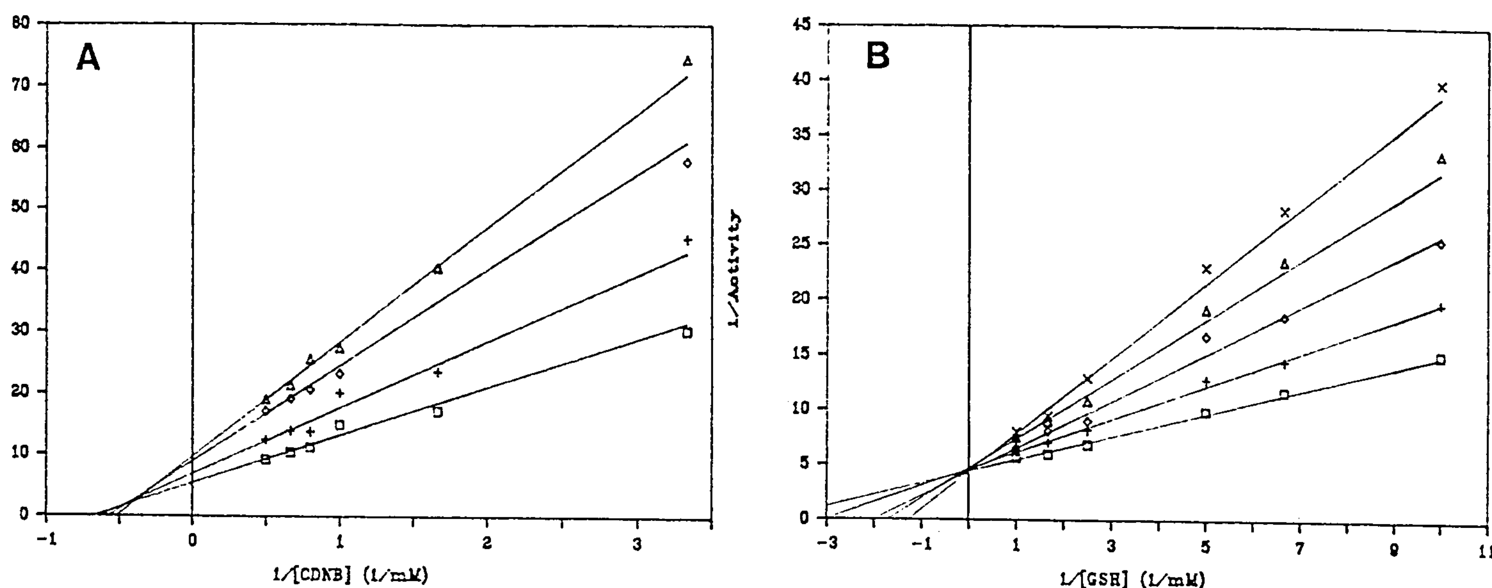


Fig. 6.4: Double-reciprocal plots of GST ρ inhibition by S-(2,4-dinitrophenyl)GSH as a function of (A) CDNB (GSH = 0.3 mM) and (B) GSH (CDNB = 1.0 mM) variation. Concentrations of the fixed inhibitor are: no inhibitor (□), 15 μM inhibitor (+), 30 μM inhibitor (◇), 45 μM inhibitor (△), and 60 μM inhibitor (×).
Straight lines are calculated from the fit of the data points to the Michaelis-Menten equation. Activity is shown as $\Delta A_{340\ nm}$/min.

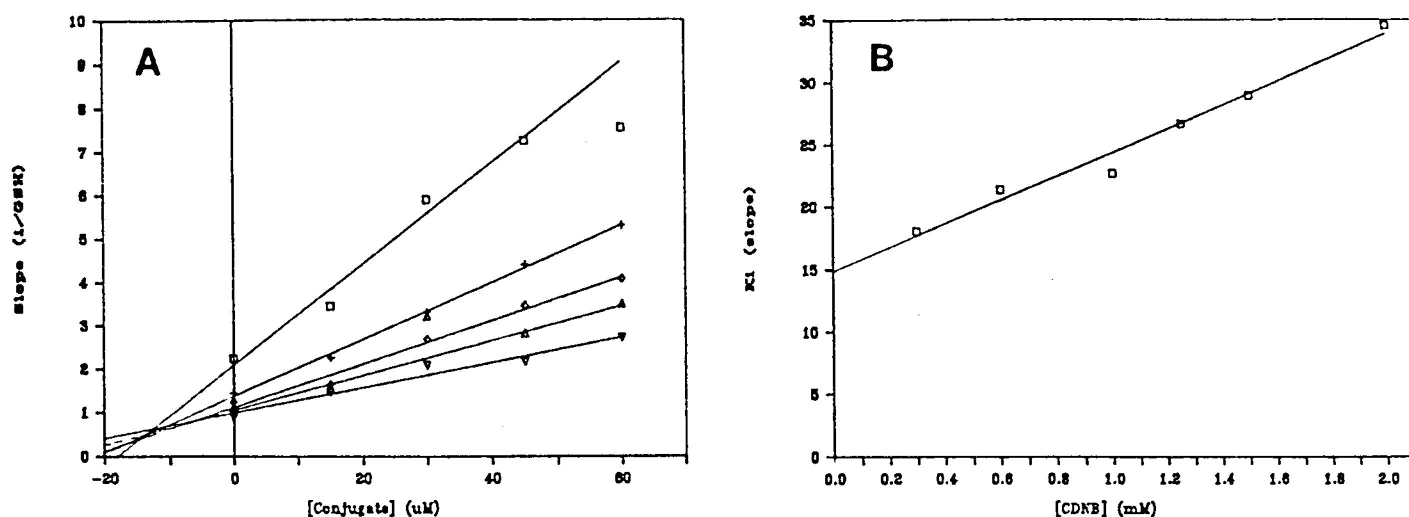


Fig. 6.5: Replots of kinetic parameters obtained with the data in fig. 6.4. (A) $Slope_{1/GSH}$ vs [conjugate] at different concentrations of CDNB and (B) $K_{i(slope)}$ vs [CDNB].
The CDNB concentrations used were 0.30 mM (□), 0.60 mM (+), 1.00 mM (◇), 1.25 mM (△), and 2.00 mM (▽).

This finding was substantiated by fitting the data to the relevant rate equations (Table 6.3). The random rapid equilibrium mechanism containing an enzyme-CDNB-product dead-end complex was found to fit the data better than a random rapid equilibrium mechanism without a dead-end complex, as indicated by better R^2 and residual sums squared values (Table 6.3). An ordered BiBi random system with product inhibition parameters could not be fitted due to the complexity of the rate equation (Segel, 1975).
The inhibition constant (K_i) determined for S-(2,4-dinitrophenyl)GSH was calculated to be $1.7 \pm 0.4 \times 10^{-5}$ M from the replots (Fig. 6.5) and $1.55 \pm 0.44 \times 10^{-5}$ M by non-linear regression (Table 6.3). The alternative product of the reaction, chloride, was devoid of inhibitory action at concentrations up to 100 μM (Fig. 6.6).

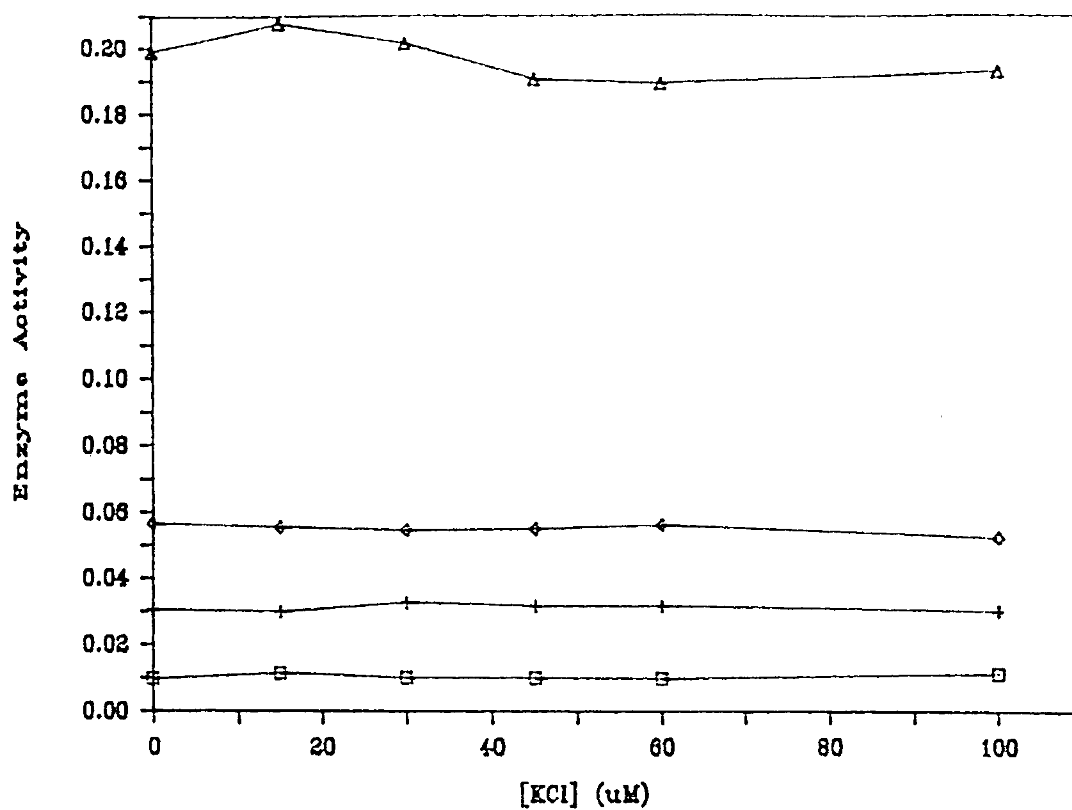


Fig. 6.6: Effect of chloride on GST ρ activity at different substrate concentrations: 0.25 mM CDNB, 0.05 mM GSH (□); 0.25 mM CDNB, 1.00 mM GSH (+); 2.00 mM CDNB, 0.05 mM GSH (◇); 2.00 mM CDNB, 1.00 mM GSH (△).

Table 6.3: Kinetic parameters obtained by fitting rate equations to initial rates in the presence of product inhibition (Segel, 1975).

Rapid equilibrium random bibi mechanism with no dead-end inhibition

$$\frac{v}{V_{max}} = \frac{[A][B]/\alpha K_A K_B}{1 + [A]/K_A + [B]/K_B + [P]/K_P + [A][B]/\alpha K_A K_B}$$

Fitted values:

V_{max}= 0.44 ± 0.03
α= 2.35 ± 1.37
K_{CDNB}= 0.44 ± 0.19
K_{GSH}= 0.15 ± 0.06
K_P= 7.6 ± 2.5 x 10^{-3}
RSS= 6.42 x 10^{-3}
R^2= 0.9815

Rapid equilibrium random bibi mechanism with dead-end enzyme-CDNB-conjugate complex

$$\frac{v}{V_{max}} = \frac{[A][B]/\alpha K_A K_B}{1+[A]/K_A+[B]/K_B+[P]/K_P+[A][B]/\alpha K_A K_B+[B][P]/\gamma K_B K_P}$$

Fitted values:

V_{max}= 0.48 ± 0.038
α= 2.03 ± 0.99
K_{CDNB}= 0.62 ± 0.22
K_{GSH}= 0.17 ± 0.05
K_P= 1.55 ± 0.44 x 10^{-3}
γ= 3.09 ± 1.49
RSS= 5.11 x 10^{-3}
R^2= 0.9853

Initial rates were fitted to the above equations by non-linear regression using the BMDP statistical analysis program.
Enzyme rates are shown as $\Delta A_{340\ nm}$/min; Michaelis constants (K) are given as concentrations (mM).
A = GSH; B = CDNB; P = Conjugate of CDNB/GSH reaction (mM);
RSS = Residual sums squared.

It is concluded that GST ρ has a random sequential rapid equilibrium mechanism with a dead-end enzyme-CDNB-conjugate inhibition complex. The Michaelis constants for the substrates CDNB and GSH were 0.70 ± 0.11 and 0.12 ± 0.02 mM, respectively, with α value of 2.0 ± 0.4. The latter value for α indicates that the binding of one substrate decreases the affinity of the enzyme for the second substrate. The enzyme is inhibited by the conjugated product but not by chloride, the second product of the reaction.

Miscellaneous Results:

6.3 Purification of Human Erythrocyte GST ρ (rho).

GST ρ was purified from the blood of apparently healthy individuals in a multi-step process. The enzyme preparations used for binding and kinetic studies were purified to specific activities of 30.6 and 42.7 μmol.min^{-1}.mg protein^{-1} (2.88 and 9.9 x 10^{-6} M enzyme), respectively, in two separate purifications.
The elution profile of erythrocyte GST ρ on a weak anion-exchange HPLC column is shown in Fig. 6.7. Both enzyme activity (Fig. 6.7) and absorbance analysis (not shown) indicated one major elution peak with other potential enzyme peaks at less than 5% of the total enzyme activity.
Since more than 50% of the loaded enzyme activity was lost on HPLC and purity of the sample was not significantly increased, this step was only used as analytical indicator of enzyme homogeneity in the purification process.

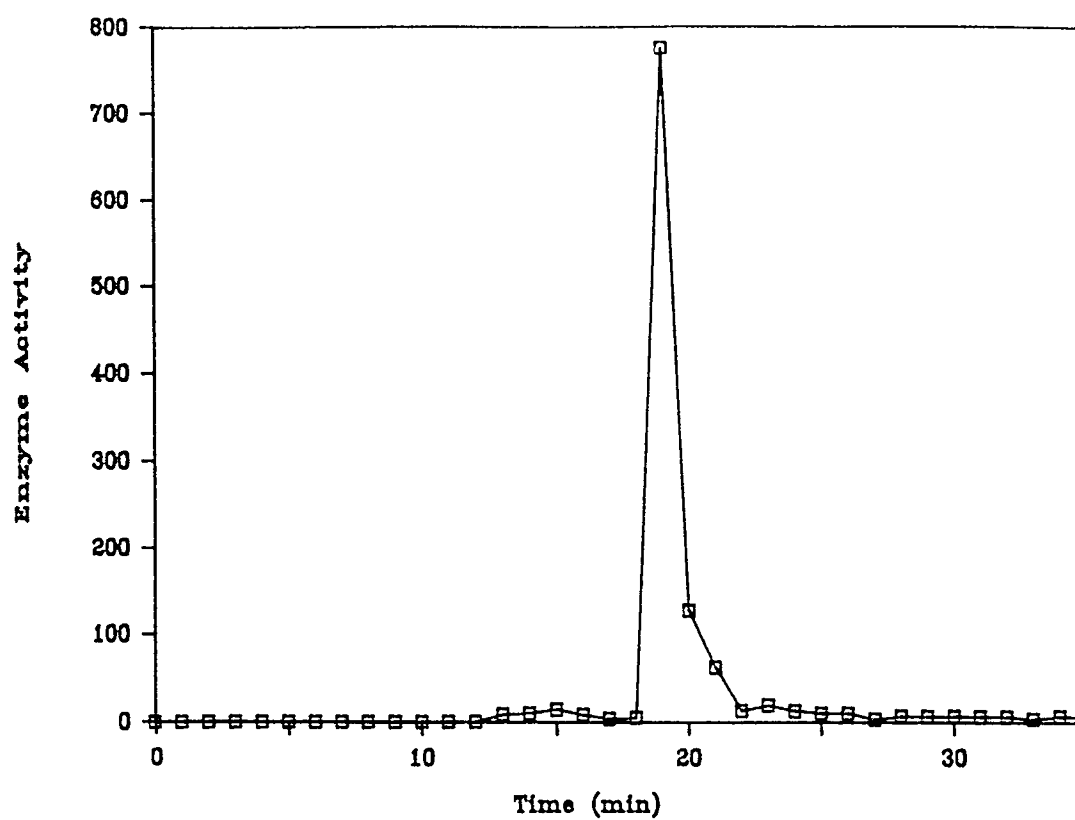


Fig. 6.7: Chromatography of GST ρ on a weak anion-exchange Synchropak AX 300 HPLC column. Enzyme activity was measured with CDNB.

6.4 Linearity of the Multistat Centrifugal Analyzer.

The CDNB assay has previously been shown to be linear at least up to 0.200 A_{340} nm/min for one min on the centrifugal analyzer (R.D. Goold, personal communication) and this has been confirmed (Fig. 6.8). This instrument was only used for the analysis of the kinetic mechanism of GST ρ.

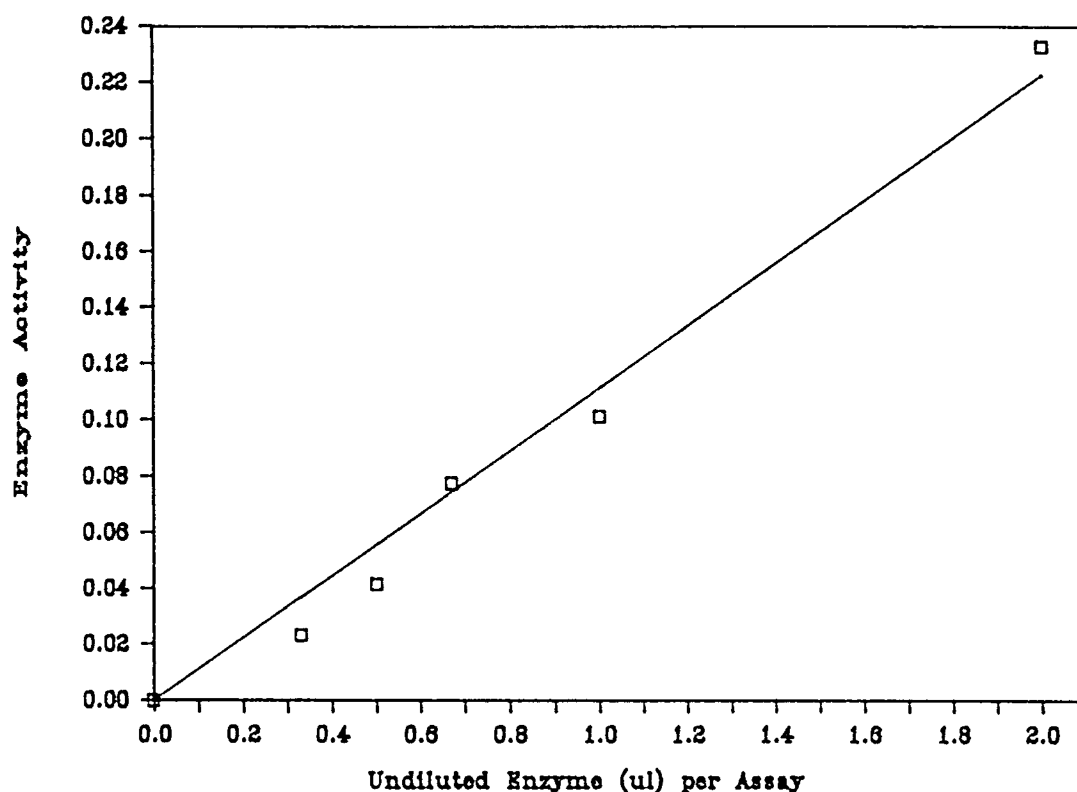


Fig. 6.8: Linearity of the CDNB assay on a Multistat centrifugal analyzer. The total assay volume was 200 μl: 0.1 M phosphate buffer containing CDNB (2% DMSO; 180 μl), 1.0 mM GSH (10 μl) and diluted GST ρ (10 μl). Measurements were started 10 sec after mixing of the sample with a total of 12 measurements at 5 second intervals at 340 nm in 0.5 cm pathlength cuvettes. Data points were fitted to a straight line by linear regression.
Activity is shown as ΔA_{340} nm/min and corrected for a 1 cm pathlength cuvette. Enzyme activity of the undiluted sample was 2.0 ± 0.4 μmol.min^{-1}.

Microperoxidases: Binding and Inhibition of GST p (rho).

7.1 Purification of Microperoxidases.

The preparation of microperoxidases by the enzymatic digestion of cytochrome *c* has in the past utilized various column purification and concentration procedures, with the resultant disadvantage that long time periods of up to 8 days were required (Peterson *et al*, 1983). We have used reverse phase HPLC chromatography as an analytical and preparative technique to purify MP-6, MP-8, MP-9 and MP-11 within 2 days.
The tryptic digestion of MP-11 to MP-8 was efficient with respect to heme peptide (≥ 95%) and the product was essentially free of non MP-8 heme by-products (Fig. 7.1). The digestion was temperature-dependent and complete within 30 min at 40 °C (Fig. 7.2) at the concentrations of MP-11 and trypsin used (see Section 2.11). MP-8 formation obeyed pseudo-first order kinetics (Fig. 7.2) which, assuming a Michaelis-Menten mechanism, indicated that the MP-11 concentration used was much lower than the Michaelis constant (K_m) for this peptide with trypsin. Arrhenius behavior was observed for the temperature variation of k_{obs}, with an apparent activation energy (E_a) of 16.2 kcal.mol^{-1} (Fig. 7.3). The further purification of MP-8 by HPLC or affinity chromatography on BSA- or HSA-Sepharose (Wilchek, 1972) to remove trypsin and non-heme contaminants could be completed within one day and the entire process could thus be concluded in two days (including lyophilization).
All subsequent microperoxidase digestions were performed at 40 °C. MP-9 samples could not be purified completely in terms of non-heme peptide contaminants, although they were pure with respect to heme content (Fig. 7.1).

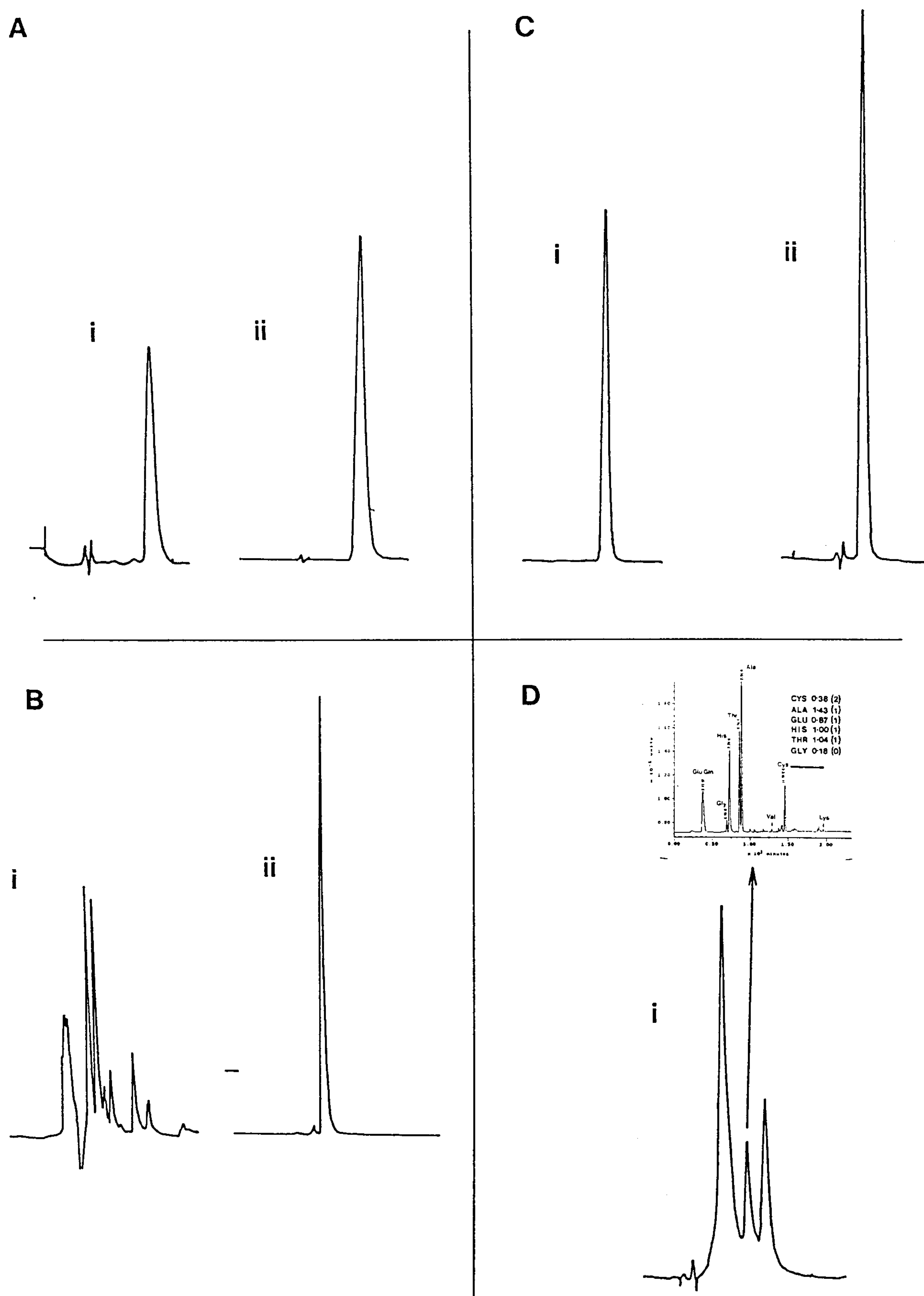


Fig. 7.1: HPLC elution profiles of microperoxidases: (A) MP-8, (B) MP-9, (C) MP-11, and (D) Nagarse digestion of cytochrome c (including the amino acid composition of the second peak). Absorbances were measured at (i) 398 nm and (ii) 210 nm. Samples were chromatographed on a Microbondpak C_{18} HPLC column with 0.1% TFA and 30% acetonitrile as the aqueous phase.

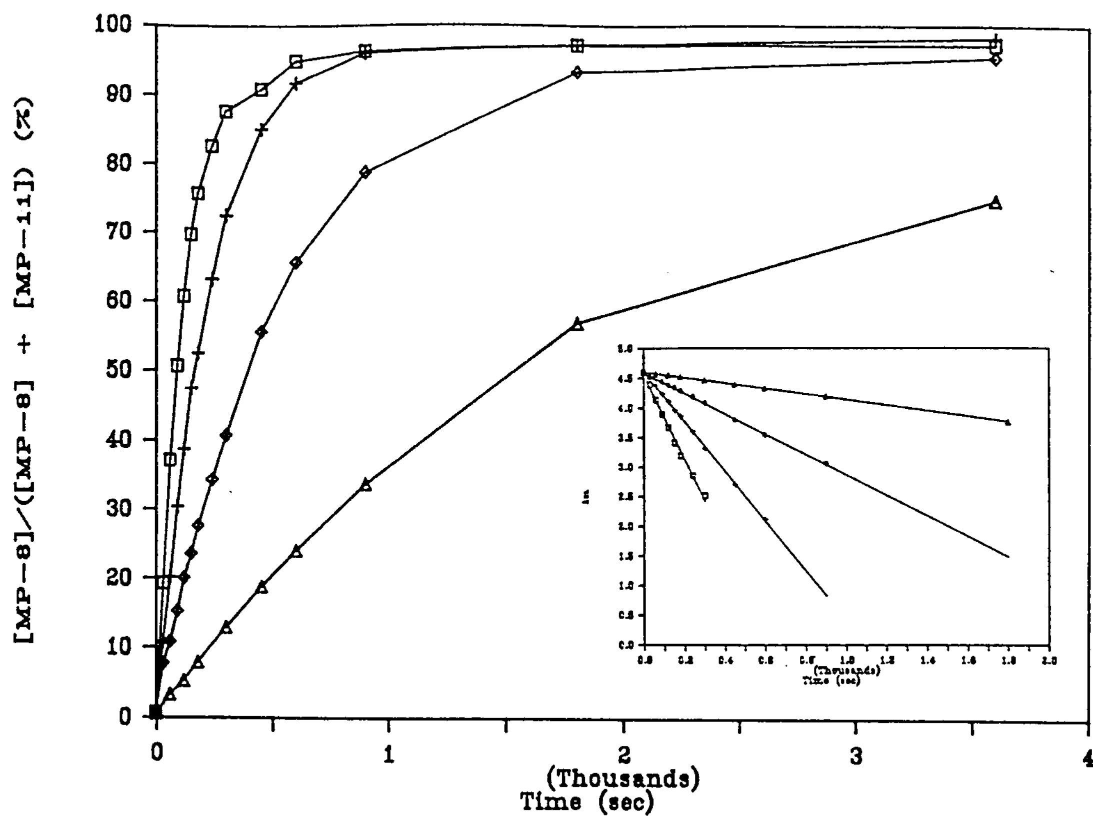


Fig. 7.2: Time-dependent trypsin digestion of MP-11 at 40 °C (□), 30 °C (+), 20 °C (◇), and 10 °C (△).
Inset: Plot of $\ln(A_t - A_\infty)$ vs time.

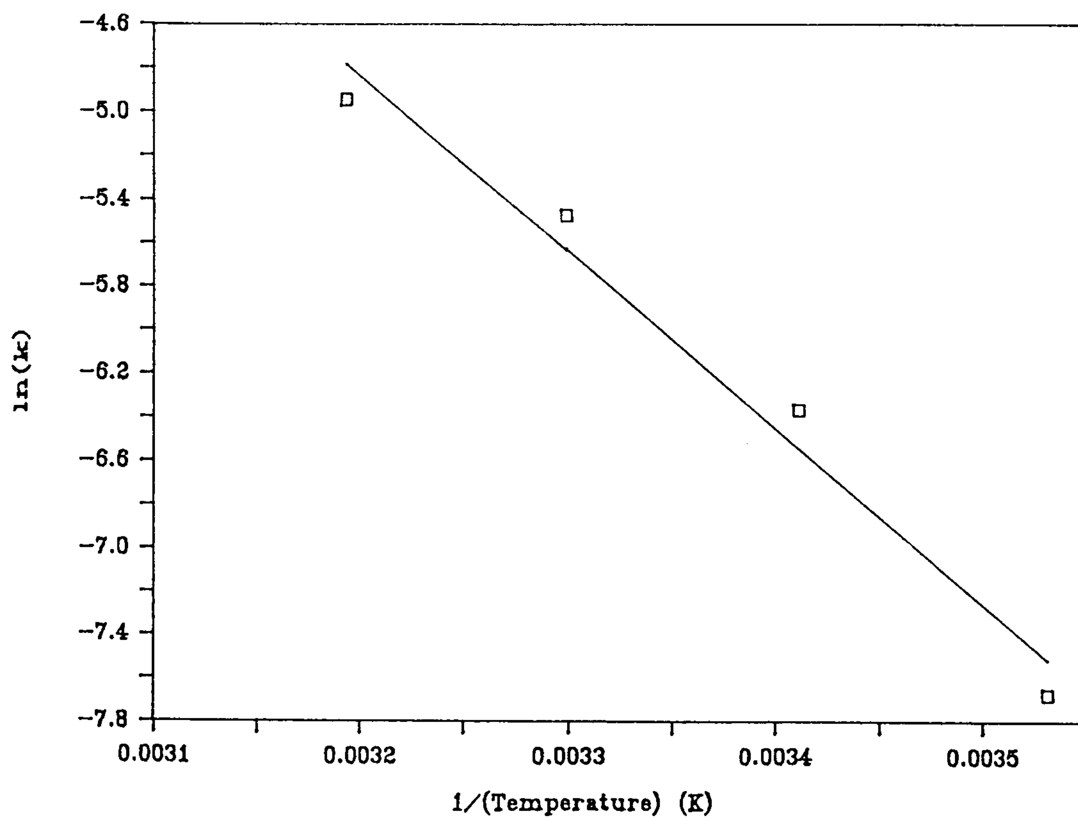


Fig. 7.3: Arrhenius plot of data shown in Fig. 7.2.

Digestion of horse heart cytochrome *c* by the non-specific protease nagarse resulted in the formation of three heme peptides which were separated cleanly on reverse phase HPLC (Fig. 7.1) but not by gel filtration chromatography on Sephadex (Fig. 7.4) (Peterson *et al*, 1983). Small quantities of the heme-peptides were collected on elution from the HPLC, lyophilized and subjected to amino acid analysis. The results of a typical digest and amino analysis of MP-6 are shown in figure 7.1 (d).

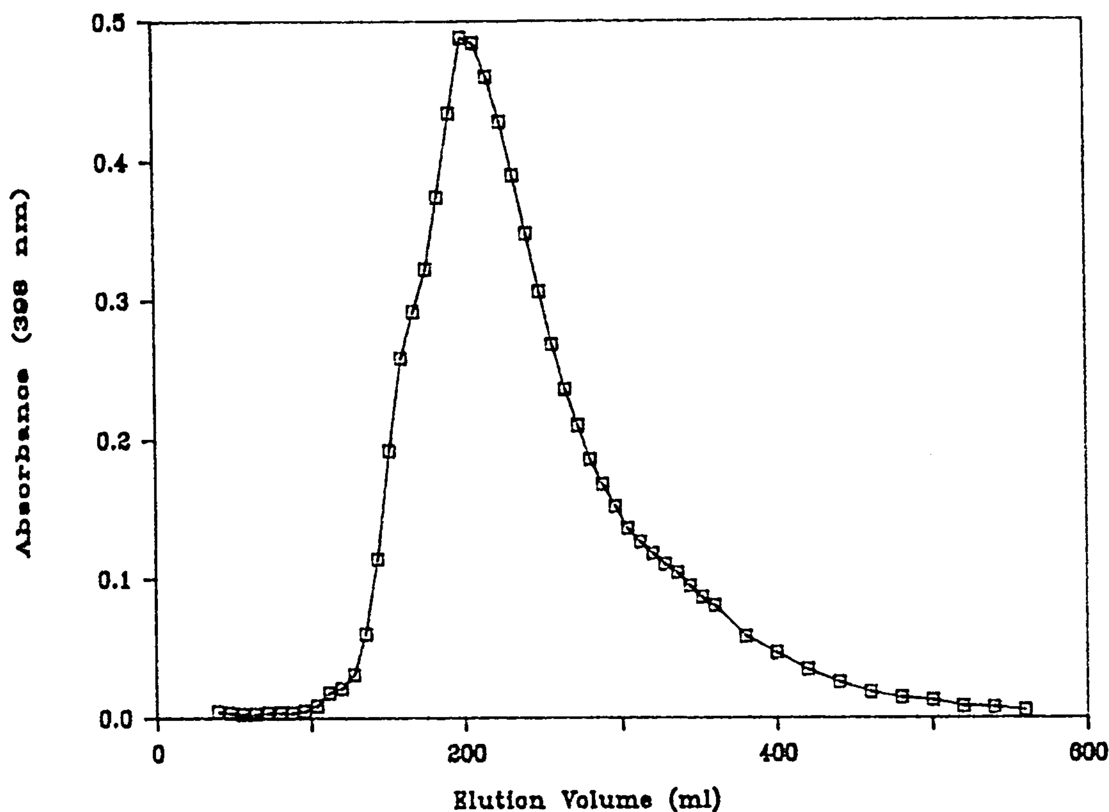


Fig 7.4: Elution profile on a Sephadex G-15 column (1.7 cm x 100 cm) of a cytochrome c nagarse digestion. The column was eluted with 2% acetic acid.

7.2 Inhibition of GST *ρ* by Microperoxidases.

Inhibition of enzyme activity by MP-8, MP-9 and MP-11 was time-dependent and displayed pseudo-first order inhibition kinetics, with rate constants of $7.5 \pm 0.7 \times 10^{-4}$, $7.7 \pm 0.8 \times 10^{-4}$ and $6.1 \pm 0.8 \times 10^{-4}\ s^{-1}$, respectively, when corrected for spontaneous loss of activity in the absence of microperoxidase (Fig. 7.5). This corresponds to an approximate half-life of 900 seconds.

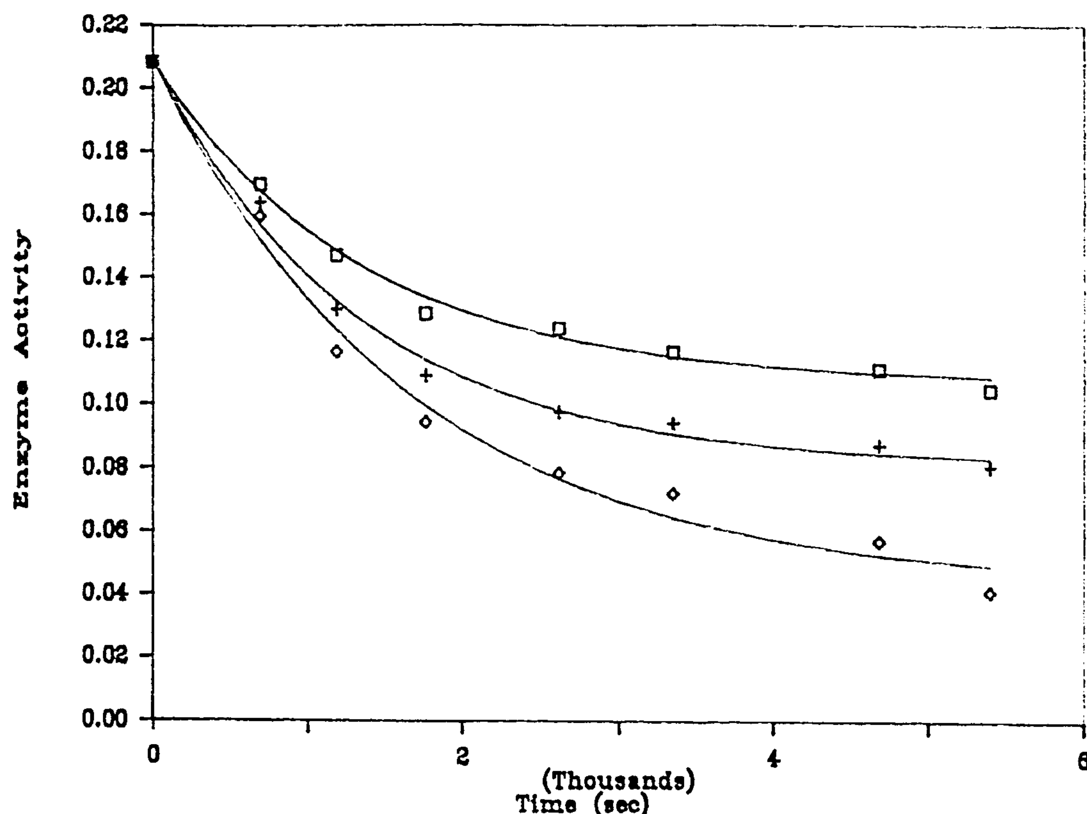


Fig. 7.5: Time-dependent inhibition of GST ρ by MP-8 (□), MP-9 (+) and MP-11 (◇) (2 x 10^{-6} M each). The enzyme (6 x 10^{-9} M) was incubated with the microperoxidases in 0.1 M phosphate buffer, pH 7.0, at room temperature (24 ± 2 °C) and enzyme activity (CDNB = 1.0 mM; GSH = 1.0 mM) measured at the relevant time points.
Curves were fitted to a pseudo first-order decay curve ($A_t = A_\infty + (A_0 - A_\infty)e^{-k_1 t}$). Activity (%) was relative to identical zero-time samples in the absence of microperoxidase.

Incubation of GST ρ with microperoxidases for 30 min before assay of enzyme activity indicated that binding of the heme peptide resulted in mixed-type inhibition kinetics with respect to CDNB (Fig. 7.6). Inhibition characteristics changed as the microperoxidase concentration was increased, as shown in double-reciprocal graphs and plots of apparent inhibition constants (Fig. 7.6). At MP-9 and MP-11 concentrations below 1 μM there was a straight line relationship between K_m/V_{max} and microperoxidase concentration. The apparent K_i values evaluated increased with microperoxidase size and values of 0.57 ± 0.09, 1.62 ± 0.16 and 3.15 ± 0.09 μM were obtained for MP-8, MP-9 and MP-11, respectively, using data obtained in the sub-micromolar region (Fig 7.7).

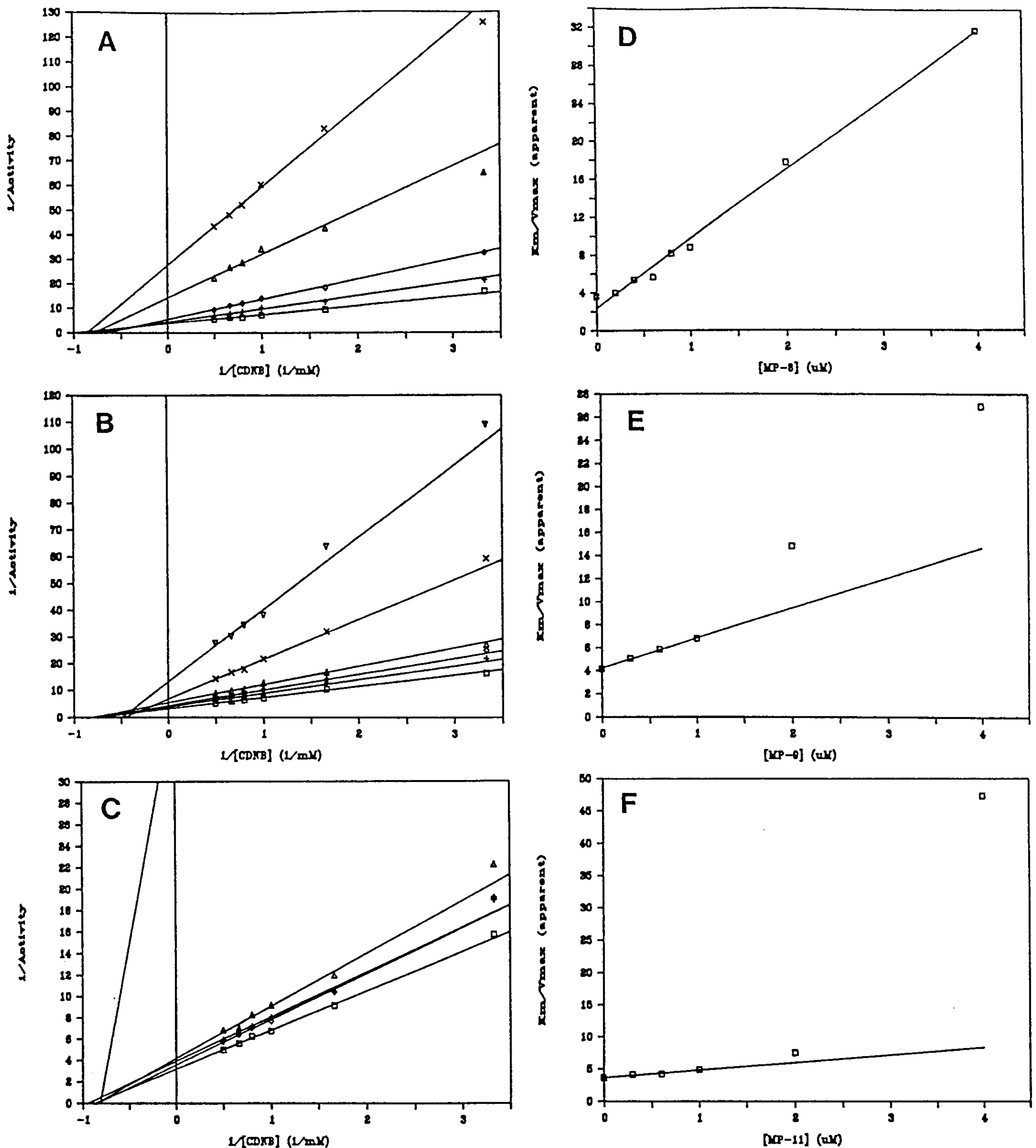


Fig. 7.6: Inhibition of GST ρ by MP-8 (A, D), MP-9 (B, E) and MP-11 (C, F) as a function of CDNB concentration (GSH = 1.0 mM). GST ρ and the microperoxidases were incubated for 30 min (0.1 M phosphate buffer, pH 7.0, 24 ± 2 °C) and enzyme activity measured. Curves were fitted to the Michaelis-Menten equation.
Replots show the relationship between the apparent K_m/V_{max} ratios and concentration of MP-8 (D), MP-9 (E) and MP-11 (F). The solid lines were obtained by fitting the data points below 1 μM to a straight line by linear regression.
Microperoxidase concentrations were:
MP-8 (Fig. 7.6 A): 0 μM (□), 0.4 μM (+), 0.8 μM (◇), 2.0 μM (△), 4.0 μM (×).
MP-9 (Fig. 7.6 B): 0 μM (□), 0.3 μM (+), 0.6 μM (◇), 1.0 μM (△), 2.0 μM (×), 4.0 μM (▽).
MP-11 (Fig. 7.6 C): 0 μM (□), 0.3 μM (+), 0.6 μM (◇), 1.0 μM (△).

7.3 Kinetics of the Binding of Microperoxidases to GST ρ.

The kinetics of the interaction of microperoxidases with heme-binding proteins can be studied spectrophotometrically (Adams *et al*, 1989); the same procedure was used to investigate microperoxidase binding to GST ρ by following time course of the absorbance decrease observed on mixing at 398 ± 1 nm (Fig. 7.7).

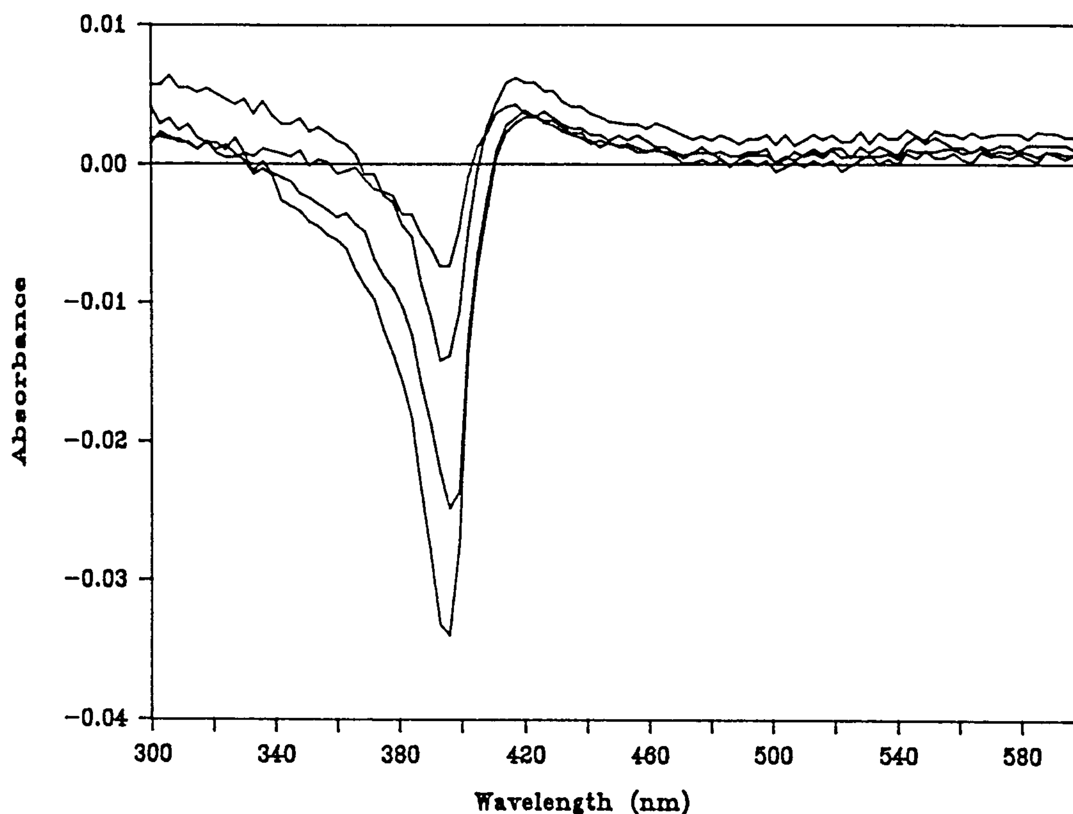
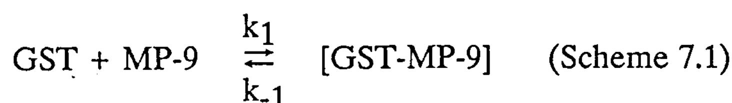


Fig. 7.7: Absorbance scans of MP-8 binding to GST ρ showing the time-dependent absorbance decrease at ≈ 398 nm. The time points are 4 min, 15 min, 25 min and 60 min.

The time-dependent binding of MP-9 to GST p followed pseudo first-order kinetics at sub-micromolar microperoxidase concentrations (Fig. 7.8). A plot of k_{obs} vs [MP-9] was a straight line (Fig. 7.9), implying a single phase reversible binding mechanism as shown in scheme 7.1 (Bernasconi, 1976), with calculated values of 4.1×10^2 $mol^{-1}.dm^3.s^{-1}$ and 1.4×10^{-4} s^{-1} for k_1 and k_{-1}, respectively, and a calculated apparent dissociation constant (K_D) of $3.4 \pm 0.7 \times 10^{-7}$ M. This value agrees well with the value of $4.5 \pm 2.0 \times 10^{-7}$ M calculated from the concentration variation of the total absorbance decrease (396 nm) obtained from the first-order fit (Fig. 7.10).



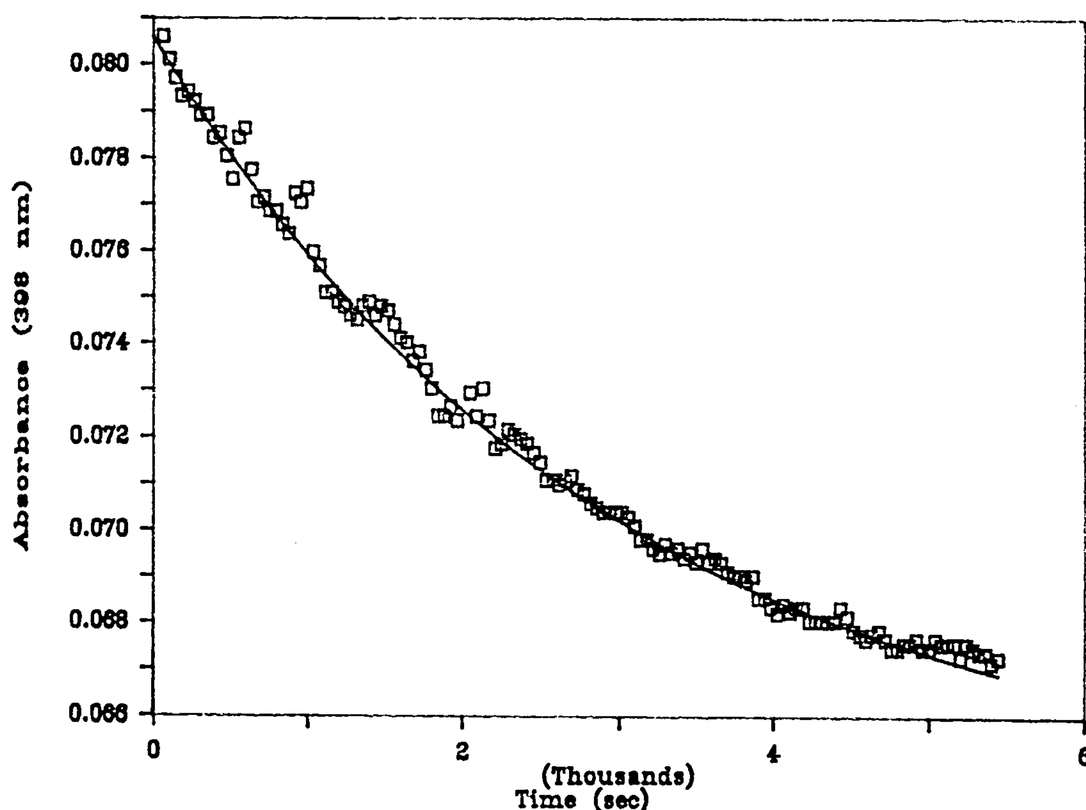


Fig 7.8: Time-dependent binding of MP-9 (0.6 μM) to GST ρ (0.03 μM). Binding was measured at 398 nm in 0.1 M phosphate buffer, pH 7.0, at 25.0 ± 0.1 °C. The curve was fitted to the equation:

$$A_t = A_\infty + (A_0 - A_\infty)e^{-k_1 t}$$

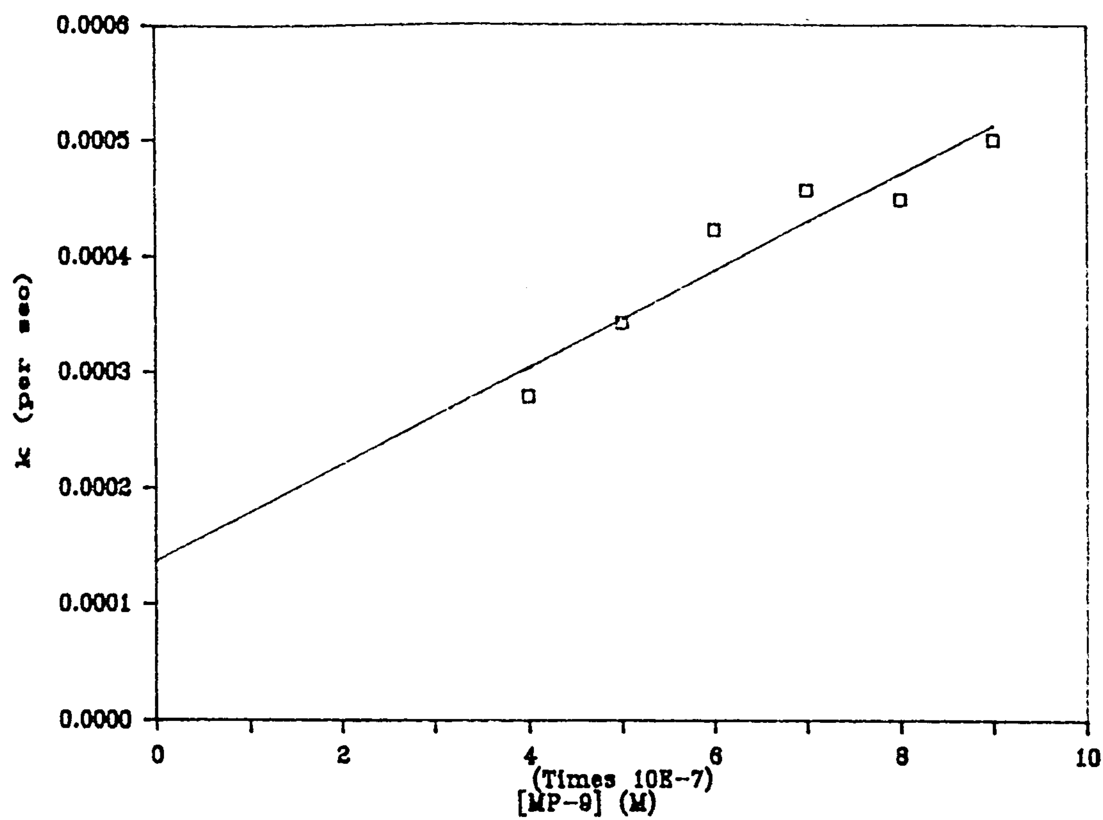


Fig 7.9: Plot of the pseudo first-order rate constants with respect to MP-9 concentration (see Fig. 7.8).

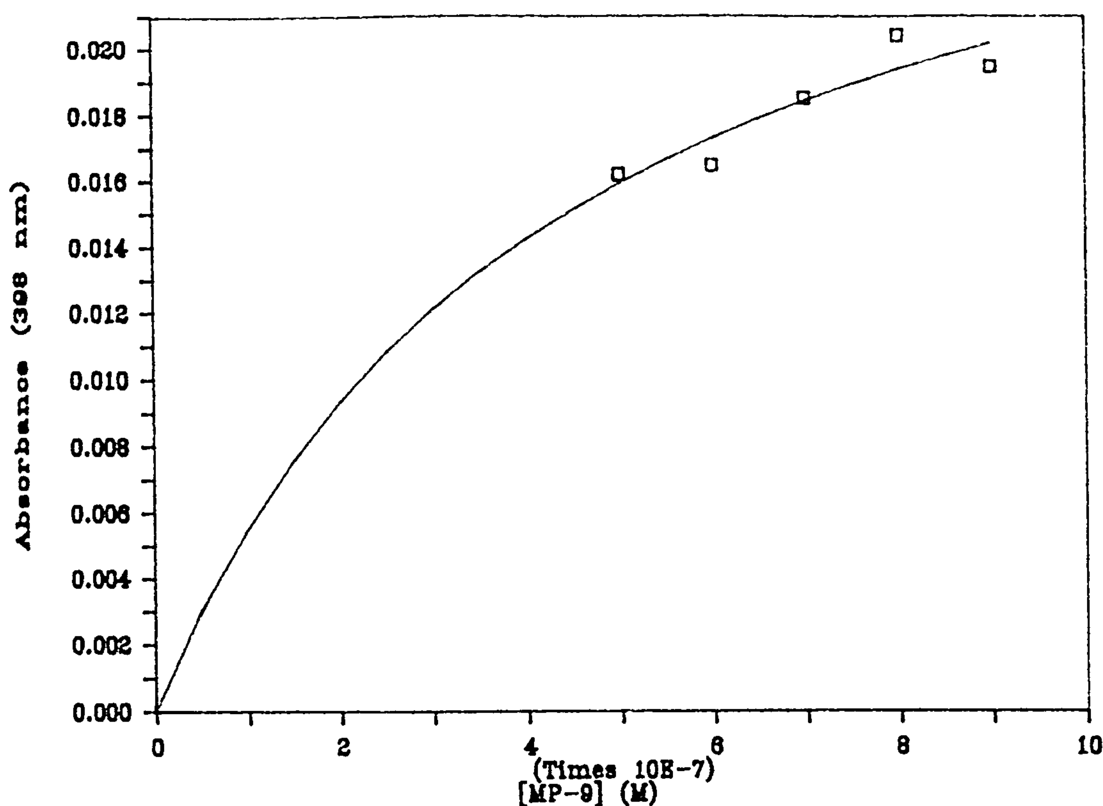


Fig. 7.10: Plot of the calculated absorbance decrease of the binding of MP-9 to GST ρ. Data was obtained as in Fig. 7.8 and fitted to a hyperbolic equation.

Incubations of GST ρ with 1.0 mM GSH for 15 min before the addition of MP-9 (0.6 μM) had no effect on MP-9 binding (Table 7.1), whereas CDNB (1.0 mM with no preincubation) abolished the pseudo first-order binding process although a small zero-order change in absorbance was observed. Pre-incubation with 15 μM bilirubin (15 min) increased the rate constant approximately 10-fold (Table 7.1), with a concomitant zero-order decrease in absorbance with time (Fig. 7.11).

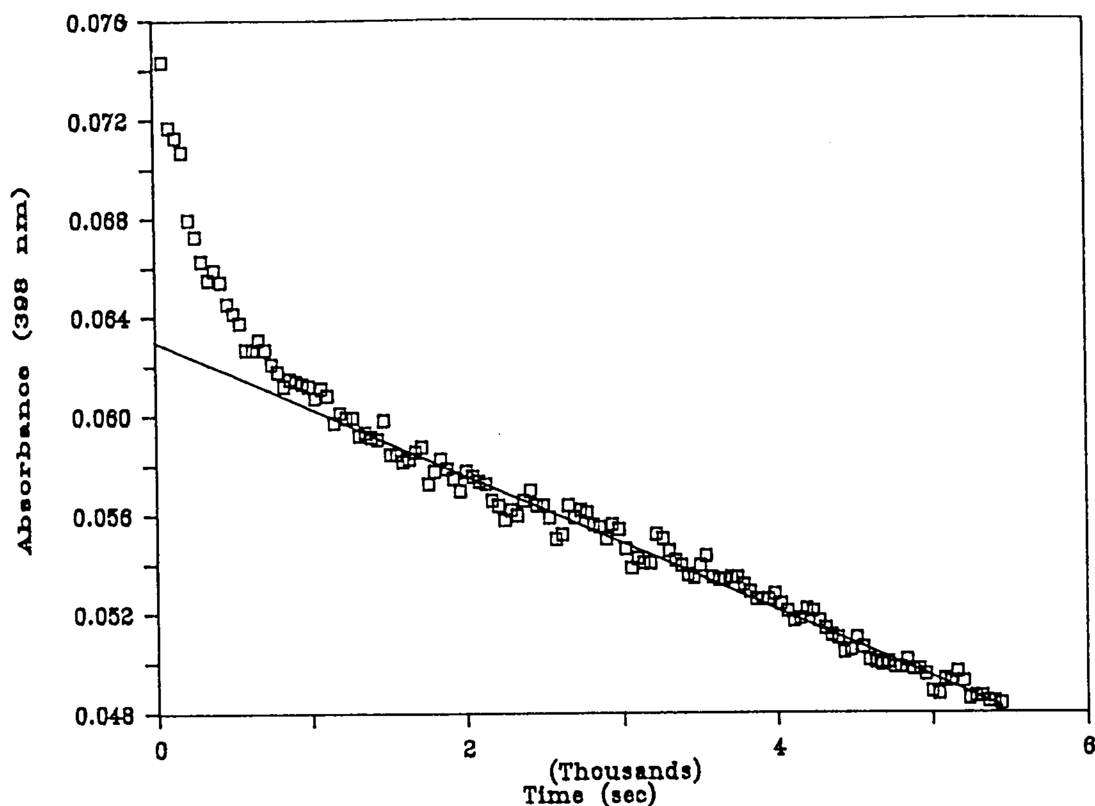


Fig. 7.11: The binding of MP-9 (0.6 μM) to GST ρ in the presence of bilirubin (15 μM). Data was obtained as in Fig. 7.8.
The line is a linear regression fit of the data at time points greater than 1000 sec.

The binding of MP-8 and MP-11 to GST ρ was also found to follow pseudo first-order kinetics and rate constants were found to be of the same order of magnitude as those observed for MP-9 (Table 7.1). However the total absorbance decrease at 398 nm varied with the various microperoxidases (Table 7.1).

Table 7.1: Pseudo first-order rate constants of microperoxidase binding to GST ρ and the effects of bilirubin and GSH addition on the rate constants.

MP (0.6 μM)	Addition	k_{obs}[a] (s^{-1})	ΔA_{398} nm[b]
MP-9	-	$4.2 \pm 0.1 \times 10^{-4}$	0.017 ± 0.0001
MP-9	Bilirubin[c]	$2.3 \pm 0.6 \times 10^{-3}$	0.010 ± 0.0006
MP-9	GSH[c]	$2.4 \pm 0.1 \times 10^{-4}$	0.023 ± 0.0003
MP-8	-	$3.1 \pm 0.1 \times 10^{-4}$	0.011 ± 0.0002
MP-11	-	$3.9 \pm 0.2 \times 10^{-4}$	0.006 ± 0.0001

Microperoxidase binding to GST ρ (3.0×10^{-6} M) was measured spectrophotomerically at 398 nm in 0.1 M potassium phosphate buffer, pH 7.0, at 25 ± 0.2 °C.

[a] Pseudo first-order rate constants were determined by fitting the data to equation: $A_t = A_\infty + (A_0 - A_\infty)e^{-k_1 t}$

[b] The total absorbance change was calculated using the least squares parameters from the best fit. Reactions were measured over 1.5 hours.

[c] The enzyme was incubated with bilirubin (15 μM) or GSH (1 mM) for 15 min before the reaction was initiated by the addition of MP-9.

Miscellaneous Results:

7.4 Concentration-Dependence of MP-8 Absorbance.

It is well-documented that the microperoxidases aggregate at micromolar concentrations (see Adams, 1990). The simplest of these, the dimers, have a lower extinction coefficient than the monomers (Aron *et al*, 1986). Therefore absorbance at 398 nm does not obey Beers law with increasing microperoxidase concentration. This deviation is demonstrated in fig. 8.9.

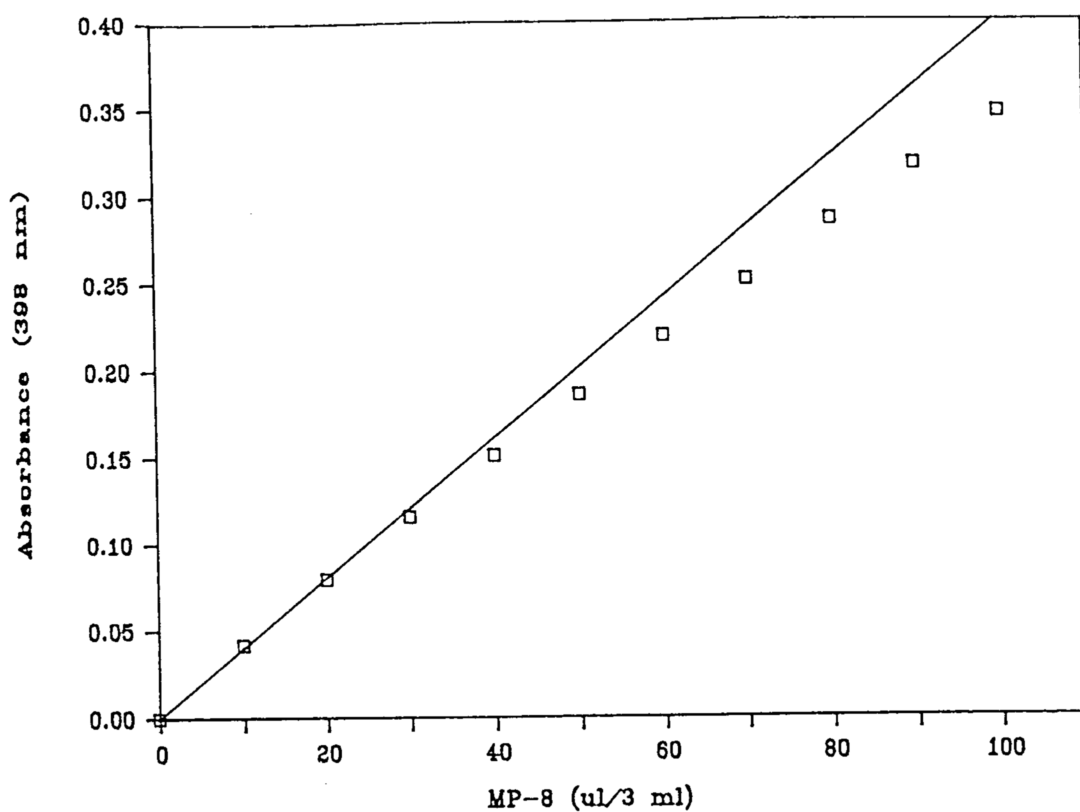


Fig. 8.9: Absorbance of MP-8 as a function of its concentration.
The indicated line is a linear regression fit to the first three points and is shown to indicate the non-linear increase in absorbance with increasing MP-8 levels.

Discussion.

8.1 The Effects of Halothane on GST Activity.

Halothane, a widely used inhalation anesthetic, is known to rarely elicit acute hepatic failure. This usually occurs after multiple exposures and is known as halothane hepatitis (Touloukian & Kaplowitz, 1981; Neuberger & Kenna, 1987). Halothane also affects various metabolic processes (Mapes, 1977; Biebuyck *et al*, 1972; Reilly *et al*, 1985), including hepatic BSP retention, the latter after multiple halothane anesthesia (Biebuyck *et al*, 1970). Since BSP retention is a function of the activity of rat GST subunit 3 (Jakoby *et al*, 1976; Mannervik & Jensson, 1982), we investigated the effect of halothane on rat hepatic GSTs *in vivo* and *in vitro*.

A single exposure of rats to halothane (1.25 MAC for 3 h) did not decrease rat hepatic GST activity towards CDNB (Table 3.1); Multiple exposures (1.25 MAC for 1 h on 3 alternate days) initially decreased and subsequently increased rat cytosolic GST activity (Table 3.2). The effect of halothane was isoenzyme-dependent. Activity towards four substrates, *ie* CDNB, DCNB, *trans*-4-phenyl-3-buten-2-one and cumene hydroperoxide, was affected. The latter three substrates are isoenzyme-specific (Boyer & Kenney, 1985; Mannervik, 1985) and reflected decreased activity of isoenzymes containing subunits 3 and 4, and GSH peroxidase activity (Table 3.2).
Other workers have shown that under similar conditions multiple, but not single, exposures to halothane increased BSP retention in isolated rat liver perfusates (Biebuyck *et al*, 1970). The binding of BSP by rat GST dimers is associated with subunit 3 (Jakoby *et al*, 1976; Mannervik & Jensson, 1982), and intrahepatic transport of this compound is facilitated by the GSTs (Boyer, 1989). The report of decreased hepatic uptake of BSP (Biebuyck *et al*, 1970) is consistent with our finding of decreased levels of GST isoenzymes containing subunit 3 (Table 3.2). Multiple exposures of rats to sub-anesthetic concentrations of halothane (0.05 MAC; 6 h a day for 9 days) has also been reported to decrease cytosolic GST activity, without affecting body weight, liver to body weight ratios or protein concentrations (Dale & Nilsen, 1984).
The decrease and subsequent increase in GST activity after multiple halothane anesthesias (Table 3.2) is similar to the induction phenomenon observed with cytochrome P-450 after a single dose of 2-allyl-2-isopropylacetamide (De Matteis, 1971). Both the microsomal cytochrome P-450 and cytosolic GST enzyme systems

are inducible in an isoenzyme-specific manner (Boyer & Kenney, 1985; Eisen, 1986). Xenobiotics commonly used to induce cytosolic GST activity include phenobarbital, 3-methylcholanthrene and *trans*-stilbene oxide (Boyer & Kenney, 1985).

Incubation of a mixture of rat hepatic GSTs with halothane, isoflurane, enflurane, methoxyflurane or DBE *in vitro* decreased activity towards CDNB (Table 3.3). Inhibition by halothane exhibited pseudo first-order kinetics ($k_{obs} = 2.2 \times 10^{-3}\ s^{-1}$; $t_{1/2} \approx 5$ min) (Fig. 3.1), with half maximal inhibition at concentrations of greater than 15 mM (Fig. 3.2). This value is significantly greater than blood concentrations of halothane (*ca* 1 mM) (Bull *et al*, 1959). Inhibition was isoenzyme-specific: Activity of GSTs 3-3, 3-4, 4-4, but not 1-1 and 1-2, was significantly reduced by halothane (Table 3.4). Human placental GST π was not inhibited by halothane, isoflurane, enflurane or DBE (Table 3.5), further confirming the isoenzyme specificity of GST inhibition.

Isoenzyme-specific inhibition of GST activity, both *in vivo* and *in vitro*, has been demonstrated for a wide variety of compounds including 1,1-dichloroethylene (Moslen & Reynolds, 1985), carbon tetrachloride (Younes *et al*, 1980), chloroform, bilirubin (Aniya & Anders, 1985a), carbon tetrachloride, bromobenzene (Aniya & Anders, 1985b), 1-chloro-2,4-dinitrobenzene (Corrigal *et al*, 1989) and 1,2-dibromoethane (Botti *et al*, 1982; Ivanetich *et al*, 1984). However, both the mechanism and specificity of GST isoenzyme inhibition vary.
Metabolites of chloroform, as catalyzed by the cytochrome P-450 enzyme system, decrease hepatic GST activity (Aniya & Anders, 1985a). In contrast halothane *per se* inhibited GST activity *in vitro* (Table 3.3). Carbon tetrachloride invoked hepatic damage (Younes *et al*, 1980), and chloroform and bromobenzene increased serum, and decreased hepatic, GST concentrations, probably as a result of hepatic GST leakage into the serum (Aniya & Anders, 1985a, 1985b). This phenomenon could be responsible for decreased hepatic GST levels *in vivo*, although this point has not been examined here. The elevation of serum GST concentrations has been proposed as a criterion of hepatic damage (Mukhtar & Bend, 1977; Aniya & Anders, 1985a, 1985b).

Inhibition of GST activity by halothane is prevented by cytosol (Table 3.6) or albumin (see Section 3.3). The albumin concentrations used in these initial experiments increased GST activity in controls . Halothane did not reduce GST activity in diluted rat hepatic cytosol (Table 3.6). Neither the *in situ* perfusion of

rat livers before preparation of cytosol nor the dialysis of cytosol reversed the protective effect of cytosol (Table 3.7). A low molecular weight compound or a component present in the blood does not therefore appear to be responsible for the protective effect. It is conceivable that cytosolic proteins protect the GSTs against inhibition: Bilirubin inhibition of GSTs is prevented by the presence of auxiliary proteins in incubations (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982, 1983), and isoenzyme 1-1 has been shown to protect other isoenzymes from bilirubin inhibition when rat hepatic cytosol was incubated with bilirubin (Fukai *et al*, 1989).

Attempts to reverse the inhibitory effects of halothane by bubbling with nitrogen were unsuccessful (see Section 3.4) suggesting that either halothane binds tightly or covalently to the GSTs . Halothane inhibition could take place by a mechanism similar to that proposed for CDNB, bromobenzene and paracetamol which form covalent bonds with the enzyme (Corrigal *et al*, 1989; Pabst *et al*, 1974; Aniya *et al*, 1988; Wendel & Cikryt, 1981). An alternative explanation is that active site solvation of GST could be facilitated by halothane, as has been proposed for both CDNB and MP-8 (Adams *et al*, 1989; Adams & Sikakana, 1990).

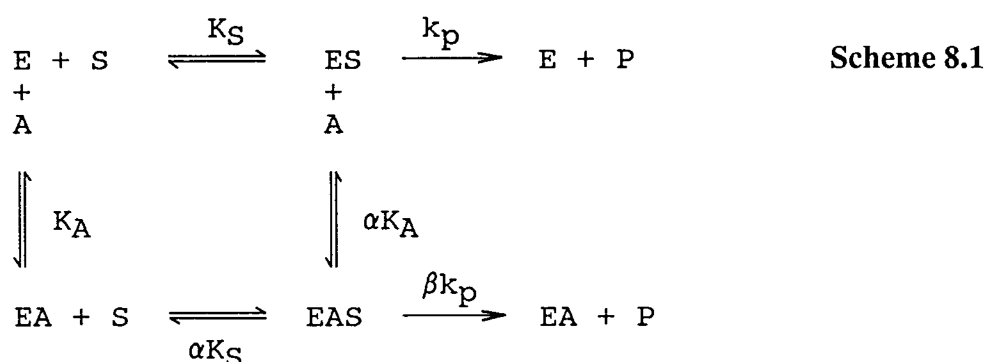
DBE, but not halothane, was conjugated with GSH upon incubation with rat liver cytosol (Fig 3.3). DBE is known to form GSH conjugates (van Bladeren *et al*, 1980), whereas GSH is only conjugated with halothane after catalysis of the latter by the cytochrome P-450 enzyme system (Cohen *et al*, 1975).

The reversible activation of GST by halothane (Fig. 3.4, Fig. 3.5, Fig. 3.6) provides evidence for halothane binding. The extent of activation is relatively low ($\leq 25\%$) and activation is reversible (see Section 3.6). Activation of cytosolic GST exhibited uncompetitive and mixed-type kinetics relative to CDNB and GSH, respectively (Fig. 3.4). However, only isoenzyme 1-2, but not isoenzymes 1-1 and 3-3, was significantly activated by halothane (Fig. 3.5, Fig. 3.6). The physiological relevance of this process is obscure.

8.2 Reversible Activation and Inhibition of GST Activity by DBE.

DBE is a nonessential, reversible activator of the rat hepatic GSTs (Fig. 4.1; Fig. 4.2; Fig. 4.3) (Segel, 1975). Cytosolic GST activity was uncompetitively activated with respect to GSH (Fig. 4.1), and activated and inhibited in an unusual manner with regard to CDNB (Fig. 4.1). The same effect was observed with isoenzymes 3-3 and 4-4 (Fig. 4.2): DBE inhibited GST activity at low concentrations of CDNB (< 0.1 mM) and activated the enzyme at high CDNB concentrations (> 0.1 mM) (Fig. 4.1 & Fig. 4.2). Isoenzymes 1-1 and 1-2 were only slightly inhibited and not activated by DBE (Fig. 4.3).

Nonessential activation can be treated kinetically as a partial mixed-type inhibition system, although with the opposite effect (Segel, 1975). In a single substrate system the pathway shown below applies:



Scheme 8.1

where E = free enzyme, S = substrate, A = nonessential activator (Segel, 1975).

The steady state rate equation for this system is:

$$v = \frac{V_{max}\dfrac{[S]}{K_S} + \beta V_{max}\dfrac{[A][S]}{\alpha K_A K_S}}{1 + \dfrac{[S]}{K_S} + \dfrac{[A]}{K_A} + \dfrac{[A][S]}{\alpha K_A K_S}}$$

Equation 8.1

Mathematical modelling of the above reaction mechanism with hypothetical values of α and β exhibits a spectrum of different modes of activation (Table 8.1). Under most conditions a simple, linear type of activation is observed. However, when α and β are greater than unity, and α is greater than β, a partial mixed-type system is encountered: The enzyme is activated at high and inhibited at low substrate concentrations (Table 8.1) (Segel, 1975). The same phenomenon being observed

with isoenzymes 3-3 and 4-4 in the presence of DBE and CDNB at relatively high concentrations of GSH (Fig. 4.2 & Fig. 4.3).

Table 8.1: The effect of different values of α and β on the type of nonessential activation[a].

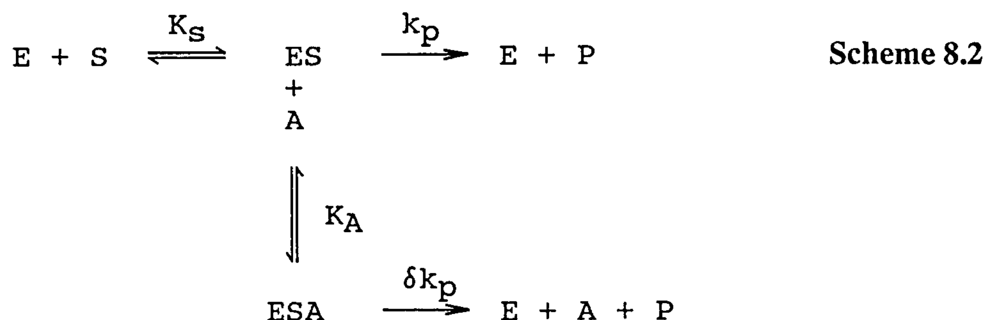
Conditions	Effect
$\alpha=1,\ \beta=1$	No inhibition or activation
$\alpha<1,\ \beta>1$	Activation at all [S]; mixed-type
$\alpha>1,\ \beta>1,\ \alpha<\beta$	Activation at all [S]; mixed-type
$\alpha>1,\ \beta>1,\ \alpha>\beta$	Inhibition at low [S], activation at high [S]; partial mixed-type
$\alpha<1,\ \beta<1,\ \alpha<\beta$	Inhibition at high [S], activation at low [S]; partial mixed-type
$\alpha>1,\ \beta>1,\ \alpha<\beta$	Activation at all [S]; mixed-type
$\alpha=1,\ \beta>1$	Activation at all [S]; mixed-type
$\alpha=1,\ \beta>1$	Activation at all [S]; mixed-type
$\alpha=1,\ \beta>1$	Activation at all [S]; mixed-type
$\alpha<1,\ \beta=1$	Activation at all [S]; competitive

[a] Different values of α and β were inserted into equation 8.1 (Segel, 1975) with hypothetical values of S, A, K_A, K_S and V_{max}. The relevant values were then plotted and analyzed.

Under conditions of high GSH and varied CDNB concentrations both α and β for the GSTs were greater than unity, as assessed from ratios of apparent V_{max} and K_m values. As α is greater than unity the binding of either the substrate (CDNB) or activator (DBE) in a random system was found to decrease the binding affinity of the enzyme for the second ligand (*ie* increasing K_A or K_S by the factor α), increasing the dissociation of the EAS complex to ES + A and EA + S (Scheme 8.1). As the constant β is greater than unity the dissociation of EAS to EA + P is also enhanced, thus increasing V_{max} by the factor β. Therefore, at low concentrations of CDNB the dissociation of the ESA complex to EA + S and ES + A predominates, suppressing activity relative to controls. At higher CDNB concentrations dissipation to the product dominates and enzyme activity is enhanced (Segel, 1975).

The GSTs were uncompetitively activated by DBE with respect to GSH (Fig. 4.1 & Fig. 4.2). In a single substrate system an uncompetitive inhibitor binds reversibly only to the enzyme-substrate complex, not to the free enzyme, forming a inactive enzyme-substrate-inhibitor combination (Segel, 1975). However, in the case of an uncompetitive activator the enzyme-substrate-activator complex cannot be inactive

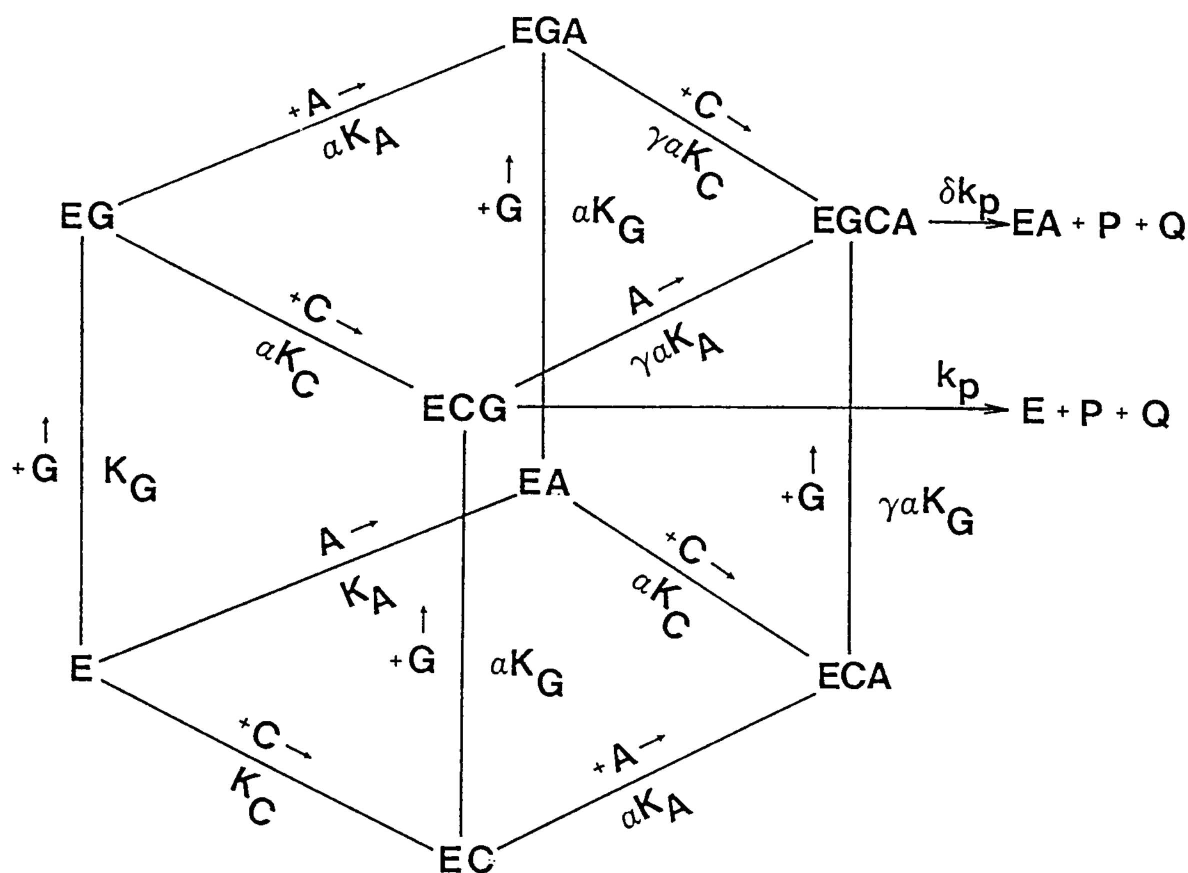
and presumably dissociates into enzyme, activator and product. It is thus hypothesized that the following equilibrium is effective:



Scheme 8.2

where E = free enzyme; S = substrate; A = activator; P = product (Segel, 1975).

Therefore V_{max} is increased as two pathways can form the product, although the rate constants for product formation are not identical. The observed decrease in K_S (Fig. 4.1 & Fig. 4.2) can be explained by a shift of the E + S = ES equilibrium towards the left. The level of free enzyme (E) could be decreased by dissociation of the ESA complex to product (P) and an inactive enzyme-activator (EA) complex. This is, however, unlikely and the increase in K_S is probably caused by a decrease in substrate concentration.
Since the GSTs are dimeric proteins with two, non-cooperative active sites per enzyme molecule (Jakobson *et al*, 1979b; Mannervik, 1985) the simple equilibrium schemes shown above (Scheme 8.1 & Scheme 8.2) are insufficient. A detailed equilibrium scheme for a random BiBi rapid equilibrium system is shown below (Scheme 8.3). GST 3-3 exhibits a random BiBi steady state mechanism (Ivanetich *et al*, 1990; Jakobson *et al*, 1977, 1979a, 1979b) and consequently scheme 8.1 does not necessarily apply. In addition, DBE metabolism has not been taken into account, and a relevant mechanistic model for a partial mixed-type inhibitor/activator system as demonstrated for DBE and the GSTs is not available at present.



Scheme 8.3: Model for a nonessential activator A (DBE) for a rapid equilibrium bireactant system. It is assumed that the binding of any two ligands has no additional effect on the binding of the third ligand. Metabolism of DBE by GST has been ignored.
C = CDNB, G = GSH
(adapted from Segel, 1975)

A comparison of the reversible and irreversible interactions of DBE with rat hepatic GSTs is shown in table 8.2. Although similar isoenzymes, comprising subunits 3 and 4, are affected by DBE, the nature of the two processes is distinct. The reversible reaction has a relatively short half-life with both inhibition and activation taking place, and the mechanism of the reaction appears to be complex. In contrast, the half-life of the irreversible process is approximately 30-fold greater than for the reversible reaction and inhibition is a pseudo first-order process.

Table 8.2: Interactions of DBE with rat GSTs.

Property:	Effect on CDNB metabolism. Reversible	Irreversible[a]
Effect on rate	Inhibition/activation	Inhibition
Half-life ($t_{½}$)	<10 sec	300 sec
K_{eq}(apparent)	Complex function	3.2 mM DBE
Isoenzymes affected	3-3, 4-4	3-3, 3-4
Isoenzymes not affected	1-1, 1-2	1-1, 1-2, 2-2

[a] (from Ivanetich *et al*, 1984)

8.3 Inhibition of GST Activity by Bilirubin and Protection by Proteins.

The time-dependent inhibition of GST activity by bilirubin is apparently a complex process involving slow conformational changes of the protein (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982). However, we hypothesized that the complex kinetics could also be the result of inhibition by CDNB or buffer, in addition to bilirubin inactivation, and possibly erroneous analysis of the relevant data, thus prompting the following investigation.

Examination of time-dependent inhibition data (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982, 1983) indicated that the extent of inhibition at infinite time points was ignored. Also, in some instances virtually all the data points are at values of greater than 60% inhibition (Vander Jagt *et al*, 1982). Replots of data at values of less than 80% inhibition, *ie* appreciably different from infinity values, shows that the inhibition kinetics are pseudo first-order (Fig. 8.1).

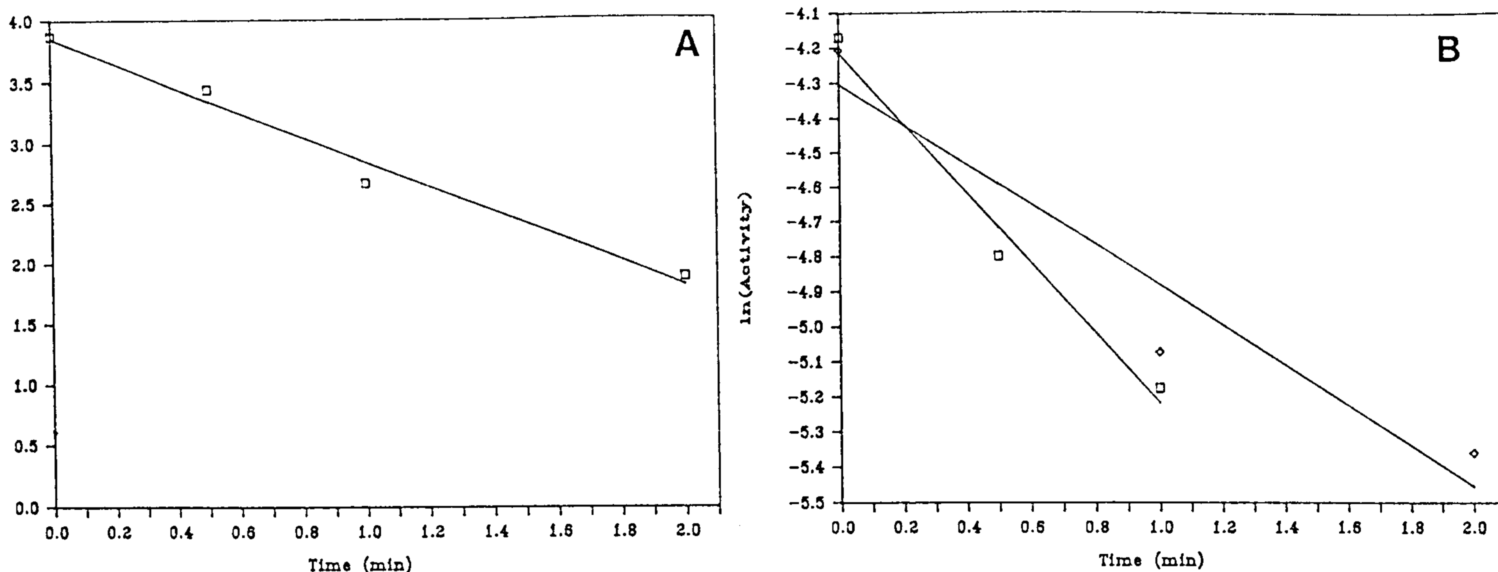


Fig. 8.1: Replots of bilirubin inhibition experiments. Data was obtained from (A) Vander Jagt et al, 1983, Fig. 7, and (B) Vander Jagt et al, 1982, Fig. 4A (□) & Fig. 4B (◇).
The lines are linear regression fits to the data.
(R^2 = 0.98, 0.98 and 0.92, respectively).

In some cases progress curves were used to determine the time-course of bilirubin inhibition (Simons & Vander Jagt, 1980; Vander Jagt et al, 1982). This method of evaluating initial velocity patterns should, however, only be performed in special cases where the equilibrium constant is high and the products of the reaction are not inhibitory. If the product does inhibit the enzyme, as occurs with the CDNB/GSH conjugate (Jakobson *et al*, 1977; Schramm *et al*, 1984), a number of progress curves are required (Cleland, 1970). Also, the inhibition at infinity time values were ignored in assessing the kinetics of inhibition.

In our study, bilirubin reversibly inhibited GST 1-2 in a pseudo-first order process (Fig. 5.1) with half-maximal inhibition at approximately 4 μM bilirubin (Table 5.1; Table 5.2). Inactivation is thus first-order with respect to enzyme (Fig. 5.1) and probably first-order with respect to bilirubin. This deduction and the calculated second-order rate constant of approximately 5.2 x 10^3 $M^{-1}.s^{-1}$ is, however, based on inhibition data at only two bilirubin concentrations (see Section 5.1).

Inhibition was reversed by dilution or by increasing pH (see Section 5.2), and was prevented by the addition of bilirubin-binding proteins (Table 5.2; Table 5.3). Inhibition by biliverdin and indocyanine green has been shown to be reversed by increased pH, although these ligands were still bound to the respective GSTs at the higher pH. Explanations for this phenomenon were that either the ionization states of the bile acids affected their ability to act as inhibitors or the enzyme-inhibitor conformation was altered (Boyer *et al*, 1984). Similar processes

could be responsible for the effect observed with bilirubin. Inactivation of GSTs 1-1 and 1-2 by bilirubin can be reversed by dilution but not by increased pH (Vander Jagt *et al*, 1982). Reversibility by dilution is consistent with the ligand binding and transport function of the GSTs *in vivo* (Sodeman & Sodeman, 1985; Boyer, 1989) and the characterization of bilirubin as a reversible GST inhibitor (Bhargava *et al*, 1978; Kamisaka *et al*, 1975; Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982, 1985; Ketley *et al*, 1975; Sugiyama *et al*, 1984).

The order of addition of components to incubations containing GST 1-2, bilirubin and albumin had a significant effect on the extent of inhibition: GST 1-2 was not significantly inhibited when bilirubin, HSA and CDNB were pre-incubated before addition of GST, while pre-incubation of GST 1-2, HSA and CDNB before bilirubin addition was found to only partially inhibit enzyme activity (Table 5.2). It is known that albumin acts as a scavenger of bilirubin in both serum and aqueous solutions (Brodersen, 1980; Simons & Vander Jagt, 1980). Thus, bilirubin pre-incubation with sufficient amounts of HSA could decrease free ligand concentrations to the extent that GST activity is not affected. In the case of bilirubin addition to both HSA and GST bilirubin probably partitions between the two proteins (Table 5.2). Thus less bilirubin is bound to the GST than in the absence of HSA, and the extent of inhibition is intermediate between that obtained in the presence and absence of HSA.

An alternative explanation is the proposed formation of GST conformations stabilized by protein-protein interactions between GST 1-2 and HSA (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982, 1983). These protein-protein interactions do not involve the formation of stable complexes and conformational states are proposed to be kinetically, and not thermodynamically, controlled. This influence of proteins on GST activity was proposed to represent "a type of enzyme memory" (Vander Jagt *et al*, 1983). A scavenger effect was rejected as levels of proteins were 100- to 1000-fold less than the bilirubin concentrations; These proteins also had no apparent effect if the bilirubin-GST complex was allowed to go to completion before the addition of protein (Simons & Vander Jagt, 1980; Vander Jagt *et al*, 1982, 1983).
However, an apparent 1000-fold excess of bilirubin is required for the complete inhibition of human ligandin (Vander Jagt *et al*, 1983). It has also been shown that GST 1-1 can protect other isoenzymes from bilirubin inhibition although a 3-fold molar excess of GST 1-1 was required to fully prevent inhibition of isoenzymes 2-2 and 3-4 (Fukai *et al*, 1989).

We have shown that albumin, aldolase and hemoglobin can bind bilirubin at high ratios, with up to 100 molecules of bilirubin apparently binding per protein molecule at concentrations of approximately 0.04 μM protein (Fig. 5.2). This ratio is relatively high, but albumin has been shown to bind up to 200 bilirubin molecules at neutral pH (Brodersen *et al*, 1972). Furthermore, bilirubin can also form both colloidal aggregates and supersaturated solutions (Brodersen & Theilgaard, 1969). Our experiments do not elucidate the mechanism of binding of high molar artios of bilirubin.

GST 1-2 inactivation was prevented at concentrations of HSA, aldolase and hemoglobin which decreased levels of bilirubin in solution to below 0.5 μM (Fig. 5.2; Table 5.3). In contrast, low concentrations of these proteins, which did not significantly decrease levels of free bilirubin, did not prevent inactivation (Fig. 5.2; Table 5.3).

The apparent contradiction observed with ribonuclease, which prevented inactivation (Table 5.3) but did not appear to bind bilirubin (Fig. 5.2), can be explained by weak binding of bilirubin to ribonuclease. The binding affinity of Sephadex for bilirubin could be higher than that of ribonuclease, resulting in the release of ribonuclease-bound bilirubin onto the Sephadex resin, thereby giving rise to the observed results. In this regard, measurement of bilirubin concentrations by Sephadex chromatography has been reported to give anomalous results (Kapitulnik *et al*, 1974) and to overestimate concentrations of free bilirubin for weak binding proteins (Kaufmann *et al*, 1973). The midpoint value of protein protection against inhibition of human ligandin by bilirubin has also been shown to be significantly higher for ribonuclease (0.5 μM) than for HSA (0.02 μM), aldolase (0.01 μM) and hemoglobin (0.003 μM) (Vander Jagt *et al*, 1983).

Further evidence cited against the role of proteins as bilirubin scavengers is the apparent inability of proteins to reverse the inhibitory process (Vander Jagt *et al*, 1983). This anomaly can be resolved by a very slow rate of bilirubin release from proteins as a result of the hydrophobic nature of the bilirubin molecule.

A similar phenomenon has been observed with the binding of the heme peptide MP-11 to HSA in aqueous solutions as a model for heme/protein interactions*. The forward rate constant for MP-11 binding to HSA (k_1) was not altered significantly by increasing the hydrophobicity of the solution with methanol (20% v/v), whereas the off rate constant (k_{-1}) was increased approximately 400-fold. We hypothesize that MP-11 binds to a sterically hindered hydrophobic

* Adams & Thumser, in preparation.

site on HSA and therefore the forward rate constant (k_1) is not affected by the hydrophobicity of the solution. Methanol, postulated as being of higher dielectric constant than the environment of the binding site, is hydrophobic enough to enter the hydrophobic binding domain and increase the effective dielectric constant (thus decreasing the hydrophobicity) of the binding site. This results in destabilization of the MP-11··HSA complex, increasing the reverse rate constant (k_{-1}). A similar process could take place with bilirubin binding to GST: A stable GST··bilirubin complex is formed and dissociation is not affected by addition of proteins as these have no direct access to the bilirubin binding site. The proteins only decrease bilirubin concentrations in solution, resulting in a constant, but slow, leaching process of bilirubin from the GST.

We propose that the protective effect of proteins is the result of a "scavenger" system which decreases the effective concentration of bilirubin in solution. More complicated explanations such as "enzyme memory" are not required to rationalize this phenomenon.

8.4 The Kinetic Mechanism of GST ρ (rho).

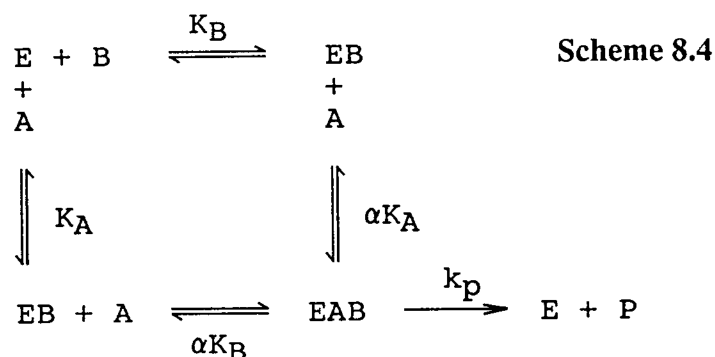
The human placental π and erythrocyte ρ GST isoenzymes are similar in terms of amino acid composition, apparent kinetic constants, substrate specificities, molecular weight and immunological cross-reactivity, and it has therefore been speculated that these two isoenzymes are either very closely related or identical (Guthenberg & Mannervik, 1981). The kinetic mechanism of the placental π isoenzyme has been demonstrated as being random sequential BiBi rapid equilibrium with dead-end enzyme-CDNB-product complex formation (Ivanetich & Goold, 1989). However, the kinetic mechanism of the human erythrocyte ρ isoenzyme has yet to be elucidated.

The kinetic mechanism of GST ρ was analyzed by determining initial rates of conjugation at various concentrations of the substrates CDNB and GSH (Segel, 1975; Cleland, 1970). Preliminary analysis of the data fitted to the Michaelis-Menten equation indicated a random sequential BiBi rapid equilibrium mechanism (Fig. 6.1; Table 6.1), Michaelis constants (K_m) for both substrates lies in the sub-millimolar range and the binding of one substrate decreases affinity for the other (Fig. 6.1) (Segel, 1975). The data was fitted to various bimolecular kinetic mechanisms, the rate equation for a random steady state mechanism was

found to display the lowest residual sums squared value (Table 6.2). However, further analysis of the relevant [substrate]/velocity plots exhibited non-random distribution of the experimental versus predicted rates, especially at low substrate concentrations (Fig. 6.2). Thus there existed a possibility that the random steady state mechanism was either an overfit of the data, or the low residual sums squared was an anomaly emanating from the limited substrate concentration range utilized. Biphasic Lineweaver-Burke plots and non-Michaelis-Menten kinetics would be predicted over a wide range of substrate concentrations for a random steady state mechanism (Segel, 1975; Cleland, 1970). Initial rate plots for supplementary experiments over a wider range of GSH concentrations were hyperbolic (Fig. 6.3), the data thus fitting the Michaelis-Menten mechanism and a random steady state mechanism was rejected (Segel, 1975; Cleland, 1970).
Examination of initial rate plots and non-linear regression (Fig. 6.2; Table 6.2) provided evidence for either a random rapid equilibrium or an ordered steady state mechanism. It is not possible to discriminate between these two mechanisms by initial rate studies alone as the relevant rate equations are mathematically equivalent, product inhibition studies were thus required to determine the true exact kinetic mechanism (Segel, 1975).

The CDNB/GSH conjugation product was prepared and its effect on initial rates at different substrate concentrations elucidated. The conjugate inhibited the enzyme in a competitive and mixed-type manner with respect to GSH and CDNB (Fig. 6.5), the K_i being of the order of approximately 16 ÌM (Fig. 6.6; Table 6.3). Further replots of the data showed that the value of $K_{i(slope)}$ was not independent of substrate concentration, *ie* the data points were not parallel to the x-axis (Fig 6.6), pointing to a random mechanism (Segel, 1975).

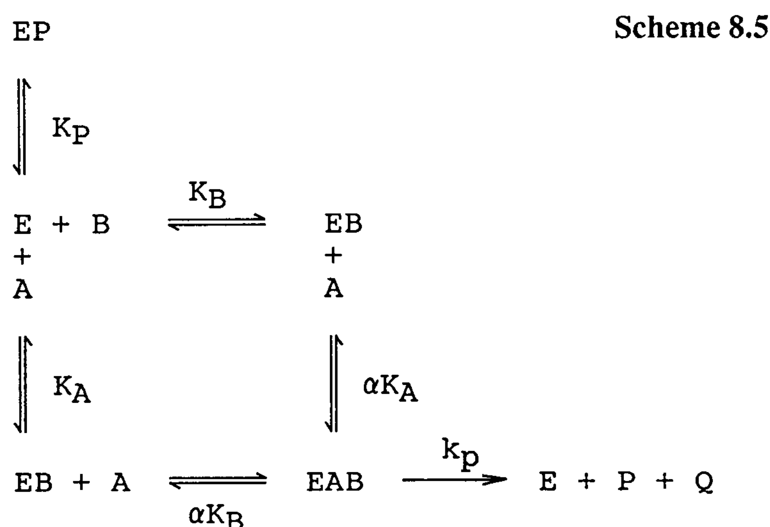
In a random sequential system the substrates are bound and products released in a arbitrary manner, but both the substrates must bind before any products are released. Binding of the first substrate affects the dissociation constant of the second substrate by a constant α, the complete process described by the following scheme:



where E = free enzyme; A, B = substrates of the enzyme; P = product of the enzyme-catalyzed reaction (Segel, 1975).

In a general random BiBi rapid equilibrium mechanism the binding of one product (P)* excludes both substrates, preventing EAP or EBP complex formation, as shown below (Scheme 8.5). The second product of the reaction (Q) can establish a EQ complex which does not exclude binding of the substrate B, generating a catalytically inactive EBQ complex. The product Q excludes A and therefore competes with the latter substrate. Increasing the concentration of B does not prevent the binding of Q and inhibition will be mixed-type or non-competitive with respect to B (Segel, 1975).

Thus, in the presence of P:



---

* Footnote: The notation of A and B as the two substrates, and P and Q as the two products, of the reaction is arbitrary and does not refer to any particular substrate or product of the GSTs at this point.

In the presence of Q:

$$
\begin{array}{ccccccc}
EQ + B & \overset{K_Q}{\rightleftharpoons} & Q + E + A + B & \overset{K_A}{\rightleftharpoons} & EA + B & & \\
\updownarrow K_B & & \updownarrow K_B & & \updownarrow \alpha K_B & & \\
EBQ & \underset{K_Q}{\rightleftharpoons} & Q + EB + A & \underset{\alpha K_A}{\rightleftharpoons} & EAB & \xrightarrow[k_p]{} & E + P + Q
\end{array}
$$

Scheme 8.6

where E = free enzyme; A, B = substrates of the enzyme; P, Q = products of the enzyme-catalyzed reaction (Segel, 1975).

The conjugation reaction of GSH with CDNB yields two products, *viz* a GSH/CDNB conjugate and a chloride ion (Pabst *et al*, 1974). The chloride ion does not inhibit the enzyme under the conditions used in the inhibition experiments (Fig. 6.7), and inactivation at higher chloride concentrations is the result of ionic strength effects (Jakobson *et al*, 1977). As the conjugate is a competitive and mixed-type inhibitor relative to GSH and CDNB (Fig. 6.5) the terms A, B, P and Q pertain to GSH, CDNB, chloride and the conjugate, respectively, in the above equilibria (Scheme 8.5 & Scheme 8.6). The EP ($E{\cdot\cdot}Cl^-$) complex does not inactivate the enzyme and scheme 8.5 can be eliminated. The mode of inhibition by the conjugate provides confirmation of an EBQ (enzyme-CDNB-conjugate) dead-end complex (Segel, 1975). This is verified by non-linear regression analysis of the kinetic results (Table 6.3).

A comparison of the kinetic constants obtained for GST ρ and GST π, which have been proposed to be similar or identical (Guthenberg & Mannervik, 1981), discloses that these two isoenzymes are comparable at the primary kinetic level (Table 8.3).

Table 8.3: Kinetic constants obtained for GST ρ and GST π with a random sequential BiBi rapid equilibrium mechanism and dead-end complex formation.

	Isoenzyme	
Parameters:[b,c]	ρ (rho)	π (pi)[a]
K_{CDNB} (mM)	0.70 ± 0.11	0.87 ± 0.07
K_{GSH} (mM)	0.12 ± 0.02	0.13 ± 0.01
K_P (mM)	0.016 ± 0.004	0.018 ± 0.003
α	2.0 ± 0.4	2.9 ± 0.27
γ	3.1 ± 1.5	8.1 ± 3.3

[a] Values for GST π were obtained from Ivanetich & Goold, 1989.
[b] Values for GST ρ are shown in Tables 6.2 and 6.3.
[c] K_{CDNB}, K_{GSH} and α were obtained by fitting the data to the random rapid equilibrium rate equation in the absence of product inhibition; K_p and γ were obtained from fits of the inhibition data to the random rapid equilibrium mechanism with dead-end enzyme-CDNB-conjugate complex formation.

Other GSTs demonstrating a random rapid equilibrium mechanism are the major bovine brain isoenzyme (Young & Briedis, 1989) and isoenzyme 1-1 (Schramm et al, 1984). In contrast, isoenzyme 3-3 displays a random sequential steady state mechanism (Jakobson et al, 1977; Ivanetich et al, 1990).

8.5 GST ρ and the Microperoxidases.

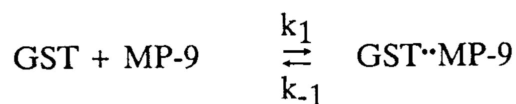
Published methods for the preparation and purification of the microperoxidases, a group of heme peptides derived from cytochrome *c*, are tedious and time consuming, requiring at least 24 hours preparative time (Peterson *et al*, 1980, 1983; Baba *et al*, 1969; Plattner *et al*, 1977). No detailed studies of the kinetics of the proteolysis reaction have been published.
The microperoxidases can be separated and identified by reverse-phase HPLC chromatography (Fig. 7.1). As MP-11 is commercially available and no known heme intermediates are produced by the tryptic digestion of MP-11 to form MP-8, investigation of the proteolytic process is relatively simple.

Analysis of the kinetics and temperature-dependence of MP-11 digestion by trypsin indicated a pseudo first-order process, with an apparent Arrhenius activation energy of 16.2 kcal.mol^{-1} (Fig. 7.2; Fig. 7.3). At 40 °C the digestion was essentially complete within two hours (Fig. 7.2). Purification of the peptide by

HPLC required less than four hours (less than 20 min per chromatography), depending on the yield of the purification, the complete process usually requiring less than six hours.
MP-8 and MP-11 were obtained at high purity (≥ 98%) by reverse-phase HPLC chromatography (Fig. 7.1), but MP-9 preparations, produced by the tryptic digestion of cytochrome *c*, were contaminated with non-heme peptides (Fig. 7.1). Nagarse digestion of cytochrome *c* yielded three heme peptides (Fig. 7.1), MP-6 could be obtained high purity by HPLC chromatography.

Microperoxidases 8, 9 and 11 inhibited GST ρ in a time-dependent process with pseudo first-order rate constants of approximately 7.5, 7.7 and 6.1 x 10^{-4} s^{-1}, respectively, corresponding to an estimated half-life of 900 seconds (Fig. 7.5). These pseudo first-order rate constants lie within the range of values obtained for the inactivation of a number of GSTs (Adams et al, 1989), although approximately 3-fold lower than the rate constant obtained for the reaction of 0.4 μM MP-8 with placental GST π (2.2 x 10^{-3} s^{-1}) (Adams et al, 1989).
Inhibition of GST ρ by the microperoxidases was mixed-type with respect to CDNB (Fig. 7.6). Values of K_i appeared to be related to microperoxidase size in the sub-micromolar range, the apparent K_i increasing from 0.57 μM (MP-8) to 1.62 μM (MP-9) and 3.15 μM (MP-11) (Fig. 7.6). Replots of K_m/V_{max} against MP concentration deviated from a straight line above concentrations of 1 μM for MP-9 and MP-11 (Fig. 7.6). This observation can almost certainly be rationalized by the dimerization characteristics of the microperoxidases as MP-9 and MP-11 are known to dimerize more extensively and in a different manner to MP-8 (Urry & Pettegrew, 1967; Urry, 1967; Peterson *et al*, 1980, 1983; Wilson *et al*, 1977). We assume that the microperoxidase dimers bind at the same site as the monomers with inhibition by the aggregates being proportionately greater than for the corresponding monomers, increasing ratios of K_m/V_{max}. As MP-8 dimerization is negligible at the sub-micromolar level (Baldwin *et al*, 1987), this effect was not apparent for MP-9 (Fig. 7.6).

The kinetics of microperoxidase binding to GST ρ can be followed spectrophotometrically at 398 nm (Fig. 7.7). Binding of MP-9 to GST ρ was monoexponential at sub-micromolar concentrations of the peptide (Fig. 7.8), thus the binding reaction appears to involve a reversible single step mechanism (Bernasconi, 1976), as shown below:



The calculated dissociation constant for MP-9 and GST ρ was approximately 4.0×10^{-7} M (Fig. 7.9, Fig. 7.10). Incubation of GST ρ with GSH did not measurably affect MP-9 binding; However, CDNB abolished MP-9 binding and bilirubin increased the rate constant for MP-9 binding approximately 10-fold (Table 7.1; Fig. 7.12).
Both CDNB and bilirubin also decreased absorbance at 398 nm in a slow zero-order process (Fig. 7.12). Zero-order kinetics are characteristic of surface reactions at substrate concentrations in excess of those required for saturation; Limiting factors being the rates of diffusion or the availability of surface sites (Bull, 1964; Capellos & Bielski, 1972). Both CDNB and bilirubin are GST inhibitors (Vander Jagt & Simons, 1980; Pabst *et al*, 1974), bilirubin having been proposed to alter the conformation of these proteins (Vander Jagt *et al*, 1982). It is plausible therefore that binding of either bilirubin or CDNB to the enzyme initiates a slow conformational change, thereby exposing more potential binding sites for MP-9, and in the case of bilirubin, intensifying the rate of binding in the second-order reaction (Fig. 7.12; Table 7.1).

Binding of MP-8 and MP-11 to GST ρ was also monoexponential, rate constants being of the same order of magnitude as for MP-9, although the total decrease in absorbance differed (Table 7.1). It can be speculated that the mechanism of binding is similar for the three microperoxidases. Inhibition would, however, also be related to the size of the peptides and their positions relative to the active site, the latter factor perhaps influencing the observed absorbance change.

The dissociation constant (K_D) of MP-9 binding to GST ρ is of the same order of magnitude as that calculated for binding of MP-8 to a kinetically slow binding site on placental GST π (Adams & Goold, 1990). MP-8 was found to be bound at two sites on GST π, *ie* a kinetically fast ($K_D = 7.2 \times 10^{-8}$ M; $t_{1/2} \approx 220$ s) and a slow site ($K_D = 5.9 \times 10^{-7}$ M; $t_{1/2} \approx 1200$ s). Binding to the fast and slow sites was eliminated by CDNB and bilirubin, respectively (Adams & Goold, 1990).
Binding of MP-8 to GST ρ is a monoexponential process with a K_D of 4.0×10^{-7} M (Fig. 7.9; Fig. 7.10), the same order of magnitude as the slow site for MP-8 binding to GST π (Adams & Goold, 1990). However, MP-8 binding at the slow site was abolish by bilirubin (Adams & Goold, 1990), while the opposite effect was observed with GST ρ. GST π has two microperoxidase binding sites per subunit, in

contrast to the single site per subunit on GST ρ, bilirubin and CDNB appearing to have disparate effects on these isoenzymes. Therefore it can be concluded that the binding site and/or binding mechanism are not the same for isoenzymes π and ρ and these proteins are probably distinct.

8.6 Conclusions.

It has been shown that halothane, an inhalational anesthetic, inactivates rat hepatic GSTs in an isoenzyme-dependent manner both *in vivo* and *in vitro*. Rats exposed to multiple doses of halothane showed a selective decrease and subsequent increase in GST activity. GST isoenzymes containing subunits 3 and 4, *viz* isoenzymes 3-3, 3-4, 4-4, were inhibited *in vitro*, and probably *in vivo*, as assessed by the substrate-specificity of GST inhibition and activation *in vivo*. Inhibition *in vitro* was a a relatively slow process with a pseudo first-order rate constant of $2.2 \times 10^{-3}\ s^{-1}$ ($t_{1/2} \approx 5$ min) and an apparent K_i of 15 mM. The latter value is substantially higher than halothane concentrations in human blood during anesthesia. Inhibition was prevented by a high molecular weight cytosolic factor, but the exact nature of the protective component was not established. In contrast to DBE, halothane was not metabolized by the GSTs, as determined by GSH conjugation. Halothane reversibly activated rat GST activity, but the physiological relevance of this observation is unknown.

DBE was a reversible, nonessential activator of rat cytosolic GSTs. The effect of DBE was unusual, activity of isoenzymes 3-3 and 4-4 inhibited or activated depending on the concentration of CDNB. These isoenzymes were uncompetitively activated with respect to GSH. DBE had no significant effect on GSTs 1-1 and 1-2. A hypothesis effects has been proposed, although a detailed analysis is not possible.

Bilirubin inhibition of GST 1-2 was concentration- ($K_i \approx 4\ \mu M$) and time-dependent, and could be reversed by dilution or increased pH. GST inactivation by bilirubin was prevented by the addition of proteins, the degree of protection dependent on both bilirubin and protein concentrations and the order of addition of constituents to incubations. We postulate that added proteins are protective as a result of "scavenging" bilirubin from solution and consider the concept of "enzyme-memory" unnecessary.

Human erythrocyte GST ρ demonstrates a random sequential BiBi kinetic mechanism with the formation of enzyme-substrate-product (enzyme-CDNB-conjugate) dead-end complex. The kinetic constants for GST ρ are similar to values published for GST π, and these two enzymes appear to be kinetically closely related at the primary kinetic level.

The procedure for preparation of the microperoxidases has been substantially improved by the use of reverse-phase HPLC. Inhibition of GST ρ by MP-8, MP-9 and MP-11 was time-dependent and pseudo first-order ($k_{obs} \approx 7 \times 10^{-4}\ s^{-1}$; $t_{1/2} \approx 900$ s), the mechanism of inhibition being mixed-type with respect to CDNB. The inhibition constant (K_i) increased with the size of the microperoxidases and also appeared to be related to dimerization characteristics of the peptides. The microperoxidases bound to GST ρ in a single-step process with a K_D for MP-9 of approximately 4×10^{-7} M. GSH had no influence on MP-9 binding, although binding was abolished by CDNB and enhanced by bilirubin. The mode of MP-9 binding to GST ρ differed from that of MP-8 to GST π. We propose that isoenzymes ρ and π, although kinetically similar, appear to show small differences at the tertiary or quaternary structural levels and are probably two discrete proteins.

References.

Adachi, Y. & Yamamoto, T.; Gastroenterol. Jpn. **22**, 34-38 (1987).

Adams & Goold; J. Chem. Soc. Faraday Trans. **86**, 1797-1801 (1990).

Adams, P.A. & Sikakana, C.N.T.; Biochem. Pharmacol. **39**, 1883-1889 (1990).

Adams, P.A., Byfield, M.P., Milton, R.C. de L. & Pratt, J.M.; J. Inorg. Biochem. **34**, 167-175 (1988).

Adams, P.A., Goold, R.D. & Sikakana, C.N.T.; Biochem. Pharmacol. **38**, 3124-3126 (1989).

Adams, P.A., Goold, R.D. & Thumser, A.E.; J. Chem. Soc. Faraday Trans. **85**, 3845-3852 (1989).

Adams, P.A.; in "Peroxidases. Chemistry and Biology" **2**, Chapter 7, eds. Everse, J. & Grisholm, M.B., CRC Press, in press (1990).

Adang, A.E.P., Brussee, J., Meyer, D.J., Coles, B., Ketterer, B., Van der Gen, A. & Mulder, G.J.; Biochem. J. **255**, 721-724 (1988).

Adang, A.E.P., Meyer, D.J., Brussee, J., Van der Gen, A., Ketterer, B. & Mulder, G.J.; Biochem. J. **264**, 759-764 (1989).

Ahokas, J.T., Davies, C., Ravenscroft, P.J. & Emmerson, B.T.; Biochem. Pharmacol. **33**, 1929-1932 (1984).

Ahr, H.J., King, L.J., Nastainczyck, W. & Ullrich, V.; Biochem. Pharmacol. **31**, 383-390 (1982).

Aitio, A. & Bend, J.R.; FEBS Letters **101**, 187-190 (1979).

Albano, E., Poli, G., Tomasi, A., Bini, A., Vannini, V. & Dianzani, M.U.; Chem. Biol. Interact. **50**, 255-265 (1984).

Ålin, P., Danielson, U.H. & Mannervik, B.; FEBS Letters **179**, 267-270 (1985).

Ålin, P., Jensson, H., Guthenberg, C., Danielson, U.H., Tahir, M.K. & Mannervik, B.; Anal. Biochem. **146**, 313-320 (1985).

Allan, L.G., Howie, J., Smith, A.F., Hussey, A.J., Beckett, G.J., Hayes, J.D. & Drummond, G.B.; Lancet **1**, 771-773 (1987).

Andersson, C., Söderström, M. & Mannervik, B.; Biochem. J. **249**, 819-824 (1988).

Aniya, Y. & Anders, M.W.; Biochem. Pharmacol. **34**, 4239-4244 (1985b).

Aniya, Y. & Anders, M.W.; Biochem. Pharmacol. **34**, 249-255 (1985a).

Aniya, Y., McLenithan, J.C. & Anders, M.W.; Biochem. Pharmacol. **37**, 251-258 (1988).

Arias, I.M., Fleischner, G., Kirsch, R., Mishkin, S. & Gatmaitan, Z.; in "Glutathione: Metabolism and Function", 175-188, eds. Arias, I.M. & Jakoby, W.B., Raven Press, New York (1976).

Arias, I.M.; Birth Defects **6**, 55-59 (1970).

Aron, J., Baldwin, D.A., Marques, H.M., Pratt, J.M. & Adams, P.A.; J. Inorg. Biochem. **27**, 227-243 (1986).

Aronsson, A.-C. & Mannervik, B.; Biochem. J. **165**, 503-509 (1977).

Asaoka, K., Ito, H. & Takahashi, K.; J. Biochem. **82**, 973-981 (1977).

Askelöf, P., Guthenberg, C., Jakobson, I. & Mannervik, B.; Biochem. J. **147**, 513-522 (1975).

Awasthi, Y.C. & Singh, S.V.; Biochem. Biophys. Res. Commun. **125**, 1053-1060 (1984).

Awasthi, Y.C., Dao, D.D. & Saneto, R.P.; Biochem. J. **191**, 1-10 (1980).

Awasthi, Y.C., Dao, D.D., Lal, A.K. & Srivastava, S.K.; Biochem. J. **177**, 471-476 (1979).

Baba, Y., Mizushima, H. & Watanabe, H.; Chem. Pharm. Bull. **17**, 82-88 (1969).

Baden, J.M., Brinkenhoff, M., Wharton, R.S., Hitt, B.A., Simmon, V.F. & Mazze, R.I.; Anesthesiology **45**, 311-318 (1976).

Baker, M.T. & Bates, J.N.; Drug Metab. Disp. **16**, 169-172 (1988).

Baldwin, D.A., Mabuya, M.B. & Marques, H.M.; S. Afr. J. Chem. **40**, 103-110 (1987).

Baldwin, D.A., Marques, H.M. & Pratt, J.M.; FEBS Lett. **183**, 309-312 (1985).

Baldwin, D.A., Marques, H.M. & Pratt, J.M.; J. Inorg. Biochem. **27**, 245-254 (1986).

Banerjee, S., Van Duuren, B.L. & Kline, S.A.; Biochem. Biophys. Res. Commun. **90**, 1214-1220 (1979).

Barnes, M.M., James, S.P. & Wood, P.B.; Biochem. J. **77**, 680-690 (1959).

Bass, N.M., Kirsch, R.E., Tuff, S.A., Marks, I. & Saunders, S.J.; Biochim. Biophys. Acta **492**, 163-175 (1977).

Beale, D., Ketterer, B., Carne, T., Meyer, D. & Taylor, J.B.; Eur. J. Biochem. **126**, 459-463 (1982).

Beale, D., Meyer, D.J., Taylor, J.B. & Ketterer, B.; Eur. J. Biochem. **137**, 125-129 (1983).

Becker, S.D. & Lamont, J.T.; Sem. Liver Dis. **8**, 183-190 (1988).

Benson, A.M. & Talalay, P.; Biochem. Biophys. Res. Commun. **69**, 1073-1079 (1976).

Benson, A.M., Talalay, P., Keen, J.H. & Jakoby, W.B.; Proc. Natl. Acad. Sci. USA **74**, 158-162 (1977).

Bernasconi, C.F.; in "Relaxation Kinetics", 11-19, Academic Press, New York (1976).

Bernstein, K.J., Verosky, M. & Triner, L.; Anesth. Analg. **64**, 531-537 (1985).

Bhargava, M.M., Listowsky, I & Arias, I.M.; J. Biol. Chem. **253**, 4112-4115 (1978).

Bhargava, M.M., Listowsky, I. & Arias, I.M.; J. Biol. Chem. **253**, 4116-4119 (1978).

Bhargava, M.M., Ohmi, N., Listowsky, I. & Arias, I.M.; J. Biol. Chem. **255**, 718-723 (1980).

Biebuyck, J.F., Lund, P. & Krebs, H.A.; Biochem. J. **128**, 711-720 (1972).

Biebuyck, J.F., Saunders, S.J., Harrison, G.G. & Bull, A.B.; Br. Med. J. **1**, 668-671 (1970).

Blackburn, W.R., Ngai, S.H. & Lindenbaum, J.; Anesthesiology **25**, 270-283 (1964).

Blumenthal, D.C. & Kassner, R.J.; J. Biol. Chem. **255**, 5859-5863 (1980).

Bogdan, K. & Grey, A.; Bureau of Toxic Substances, New York State Dept. of Health, 1-8 (1984).

Bonnett, R., Davies, J.E. & Hursthouse, M.B.; Nature **262**, 327-328 (1976).

Booth, J., Boyland, E. & Sims, P.; Biochem. J. **74**, 117-122 (1960).

Booth, J., Boyland, E. & Sims, P.; Biochem. J. **79**, 516-524 (1961).

Bora, P.S., Spilburg, C.A. & Lange, L.G.; Proc. Natl. Acad. Sci. USA **86**, 4470-4473 (1989a).

Bora, P.S., Spilburg, C.A., Lange, L.G.; J. Clin. Invest. **84**, 1942-1946 (1989b).

Börsch, G. & Schmidt, G.; Med. Klin. **12**, 450-455 (1987).

Botti, B., Moslen, M.T., Trieff, N.M. & Reynolds, E.S.; Chem.-Biol. Interact. **42**, 259-270 (1982).

Bottinger, L.E., Dalen, E. & Hallen, B.; Acta Anaesth. Scand. **20**, 40-46 (1976).

Boyer, T.D. & Kenney, W.C.; Biochem. J. **230**, 125-132 (1985).

Boyer, T.D. & Kenney, W.C.; in "Biochemical Pharmacology and Toxicology", **1**, 297-364, eds. Zakim, D. & Vessey, D.V., John Wiley and Sons, New York (1985).

Boyer, T.D., Kenney, W.C. & Zakim, D.; Biochem. Pharmacol. **32**, 1843-1850 (1983).

Boyer, T.D., Vessey D.A. & Kempner, E.; J. Biol. Chem. **261**, 16963-16968 (1986).

Boyer, T.D., Vessey, D.A., Holcomb, C. & Saley, N.; Biochem. J. **217**, 179-185 (1984).

Boyer, T.D.; Hepatology **9**, 486-496 (1989).

Boyer, T.D.; J. Biol. Chem. **261**, 5363-5367 (1986).

Boyland, E. & Levi, A.A.; Biochem.J. **30**, 1225 (1936).

Boyland, E. & Sims, P.; Biochem. J. **68**, 440-447 (1958).

Boyland, E.; in "Concepts in Biochemical Pharmacology", **28**, 548-608, eds. Brodie, B.B. & Gillette, J.R., Springer Verlag, New York (1971).

Boyland,E. & Chasseaud, L.F.; Adv. Enzymol. **32**, 173-219 (1969).

Bradford, M.M.; Anal. Biochem. **72**, 248-254 (1976).

Brandt, I., Brittebo, E.B., Kowalski, B. & Lund, B.-O.; Carcinogenesis **8**, 1359-1361 (1987).

Bray, H.G., Franklin, T.J. & James, S.P.; Biochem. J. **71**, 690-696 (1959).

Bray, H.G., James, S.P. & Thorpe, W.V.; Biochem. J. **67**, 607-616 (1957).

Bray, H.G., James, S.P. & Thorpe, W.V.; Biochem. J. **70**, 570-579 (1959).

Brehe, J.E. & Burch, H.B.; Anal. Biochem. **74**, 189-197 (1976).

Brodersen, R. & Theilgaard, J.; Scand. J. Clin. Lab. Invest. **24**, 395-398 (1969).

Brodersen, R., Funding,L., Pedersen, A.O., & Rojgaard-Petersen, H.; Scand. J. Clin. Lab. Invest. **29**, 433-446 (1972).

Brodersen, R., Honore, B., & Larsen, F.G.; Acta Pharmacol. Toxicol. **54**, 129-133 (1984).

Brodersen, R.; CRC Crit. Rev. Clin. Lab. Sci. **11**, 305-401 (1980).

Brodie, B.B., Reid, W.D., Cho, A.K., Sipes, G., Krishna, G., Gillette, J.R.; Proc. Natl. Acad. Sci. USA **68**, 160 (1970).

Bull, A.B., Rossouw, G.J., Kench, J.E. & Barnard, C.N.; S.A. Med. J. **33**, 1097-1099 (1959).

Bull, H.B.; in "An Introduction to Physical Biochemistry.", 343, F.A. Davis Company, Philadelphia (1964).

Burgess, J.R., Yang, H., Chang, M., Rao, M.K., Tu, C.P.-D. & Reddy, C.C.; Biochem. Biophys. Res. Commun. **142**, 441-447 (1987).

Burk, R.F., Trumble, M.J. & Lawrence, R.A.; Biochim. Biophys. Acta **618**, 35-41 (1980).

Capellos, C. & Bielski, B.H.J.; in "Kinetic Systems. Mathematical Description of Chemical Kinetics in Solution.", 7-8, Wiley-Interscience, New York (1972).

Carne, T., Tipping, E. & Ketterer, B.; Biochem. J. **177**, 433-439 (1979).

Chang, M., Hong, Y., Burgess, J.R., Tu, C.-P.D. & Reddy, C.C.; Arch. Biochem. Biophys. **259**, 548-557 (1987).

Chang, M., Rao, M.K., Reddanna, P., Li, C.H., Tu, C.-P.D., Corey, E.J. & Reddy, C.C.; Arch. Biochem. Biophys. **259**, 536-547 (1987).

Chasseaud, L.F.; Drug Metab. Rev. **2**, 185-220 (1973).

Chasseaud, L.F.; in "Glutathione: Metabolism and Function.", 77-114, eds. Arias, I.M. & Jakoby, W.B., Raven Press, New York (1976).

Chaykin, S.; in "Biochemistry Laboratory Techniques", 20, John Wiley and Sons, New York (1966).

Chen, W.-J., Boehlert, C.C., Rider, K. & Armstrong, R.N.; Biochem. Biophys. Res. Commun. **128**, 233-240 (1985).

Chen, W.-J., Graminski, G.F. & Armstrong, R.N.; Biochem. **27**, 647-654 (1988).

Clark, A.G. & Sinclair, M.; Biochem. Pharmacol. **37**, 259-264 (1988).

Clark, A.G., Letoa, M. & Ting, W.S.; Life Sciences **20**, 141-148 (1977).

Clark, A.G., Smith, J.N. & Speir, T.W.; Biochem. J. **135**, 385-392 (1973).

Cleland, W.W.; in "The Enzymes: Kinetics and Mechanism", **2**, 1-65, eds. Boyer, P.D., Academic Press, New York (1970).

Cohen, E., Gamiel, A. & Katan, J.; Pestic. Biochem. Physiol. **26**, 1-9 (1986).

Cohen, E.; Comp. Biochem. Physiol. **B88**, 675-680 (1987).

Cohen, E.N., Trudell, J.R., Edmunds, H.N. & Watson, E.; Anesthesiology **43**, 392-401 (1975).

Coles, B., Meyer, D., Jernstrom, B., Beland, F. & Ketterer, B.; Br. J. Cancer **48**, 145 (1983).

Combes, B. & Stakelum, G.S.; J. Clin. Invest. **40**, 981-988 (1961).

Corrigal, A.V., Bhargava, M.M., Ivanetich, K.M., Ehlers, M.R.W. & Kirsch, R.E.; Biochim. Biophys. Acta **991**, 399-404 (1989).

Costa, A.K. & Ivanetich, K.M.; Carcinogenisis **5**, 1629-1639 (1984).

Dale, O. & Nilsen, O.G.; Toxicol. Lett. **23**, 61-66 (1984).

Daly, J.W., Jerina, D.M. & Witkop, B.; Arch. Biochem. Biophys. **128**, 517-527 (1968).

Daly, J.W., Jerina, D.M. & Witkop, B.; Experientia (Basel) **28**, 1129-1149 (1972).

Danielson, U.H. & Mannervik, B.; Biochem. J. **231**, 263-267 (1985).

Dao, D.D., Partridge, C.A., Kurosky, A. & Awasthi, Y.C.; Biochem. J.; **221**, 33-41 (1984).

Davies, T.H.; Biochim. Biophys. Acta **329**, 108-117 (1973).

De Groot, H. & Noll, T.; Anesthesiology **62**, 44-48 (1985).

De Matteis, F.; Biochem. J. **124**, 767-777 (1971).

Del Boccio, G., Di Ilio, C., Casalone, E., Pennelli, A., Aceto, A., Sacchetta, P. & Federici, G.; Ital. J. Biochem. **36**, 8-17 (1987).

DePierre, J.W. & Moron, M.S.; Pharmacol. Res. Commun. **11**, 421-431 (1979).

deSmidt, P.C., McCarrick, M.A., Darnow, J.N., Mervic, M. & Armstrong, R.N.; Biochem. Int. **14**, 401-408 (1987).

Di Ilio, C., Aceto, A., Del Boccio, G., Casalone, E., Pennelli, A. & Federici, G.; Eur. J. Biochem. **171**, 491-496 (1988).

Di Ilio, C., Del Boccio, G., Massoud, R. & Federici, G.; Biochem. Int. **13**, 263-269 (1986).

Di Ilio, C., Sacchetta, P., Del Boccio, G., Casalone, E. & Polidoro, G.; Placenta **7**, 155-162 (1986).

Dienstag, J.L.; New Engl. J. Med. **303**, 102-104 (1980).

Dierickx, P.J.; Enzyme **27**, 25-32 (1982).

Dierickx, P.J.; Res. Commun. Chem. Pathol. Pharmacol. **51**, 285-288 (1986).

DiRenzo, A.B., Gandolfi, A.J. & Sipes, I.G.; Toxicol. Letters **11**, 243-252 (1982).

Dirr, H.W. & Schabort, J.C.; Biochim. Biophys. acta **957**, 173-177 (1988).

Dohn, D.R. & Anders, M.W.; Biochem. Biophys. Res. Commun. **109**, 1339-1345 (1982).

Dostal, L.A., Horton, J.K., Harris, C., Brier, D.F. & Bend, J.R.; Carcinogenesis **8**, 1601-1606 (1987).

Douglas, K.T.; Meth. Enzymol. **59**, 103-168 (1987).

Dunford, H.B. & Stillman, J.S.; Coord. Chem. Rev. **19**, 187-251 (1976).

Edmunds, H.N., Baden, J.M. & Simmon, V.F.; Anesthesiology **51**, 424-429 (1979).

Edwards, K., Jackson, H. & Jones, A.R.; Biochem. Pharmacol. **19**, 1783-1789 (1970).

Eisen, H.J.; in "Cytochrome P-450. Structure, Mechanism, and Biochemistry.", 315-344, eds. Ortiz de Montellano, P., Plenum Press, New York (1986).

Ellman, G.L.; Arch. Biochem. Biophys. **82**, 70-77 (1959).

Ernster, L., Siekevitz, P. & Palade, G.E.; J. Cell Biol. **15**, 541-562 (1962).

Fahey, R.C.; Adv. Exp. Med. Biol. **86A**, 1 (1977).

Faulder, C.G., Hume, R. & Strange, R.C.; Biochem. Soc. Trans. **14**, 896-897 (1986).

Federici, G., Di Ilio, C., Sacchetta, P., Polidoro, G. & Bannister, J.V.; Int. J. Biochem. **17**, 653-656 (1985).

Fishbein, L.; in "Potential Industrial Carcinogens and Mutagens", 240-265, eds. Fishbein, L., Elsevier, Amsterdam (1979).

Fjellstedt, T.A., Allen, R.H., Duncan, B.K. & Jakoby, W.B.; J. Biol. Chem. **248**, 3702-3707 (1973).

Fujii, K., Miki, N., Sugiyama, T., Morio, M., Yamano, T. & Miyake, Y.; Biochem. Biophys. Res. Commun. **102**, 507-512 (1981).

Fukai, F., Yatomi, S., Morita, T., Nishizawa, S., Nagai, T. & Katayama, T.; J. Biochem. **105**, 968-973 (1989).

Gandolfi, A.J., White, R.D., Sipes, I.G. & Pohl, L.R.; J. Pharmacol. Exp. Ther. **214**, 721-725 (1980).

Gibson, D.D., Hornbrook, K.G. & McCay, P.B.; Biochim. Biophys. Acta **620**, 572-582 (1980).

Giler, Sh., Ventura, E., Levy, E., Urca, I., Sperling, O. & De Vries, A.; Experientia **32**, 620-621 (1976).

Gillette, J.R.; Drug Metab. Rev. **10**, 59-87 (1979).

Gillham, B.; Biochem. J. **135**, 797-804 (1973).

Gonzales, M.C., Sutherland, E. & Simon, F.R.; J. Clin. Invest. **63**, 684-694 (1979).

Gourlay, G.K., Adams, J.F., Cousins, M.J. & Hall, P.; Anesthesiology **55**, 96-103 (1981).

Gourlay. G.K., Adams, J.F., Cousins, M.J. & Sharp, J.H.; Br. J. Anaesth. **52**, 331-335 (1980).

Gourley, G.R. & Odell, G.B.; in "Human Gastrointestinal Development", 581-621, eds. Lebenthal, E., Raven Press, New York (1989).

Grahnen, A. & Sjoholm, I.; Eur. J. Biochem. **80**, 573-580 (1977).

Graminski, G.F., Kubo, Y. & Armstrong, R.N.; Biochem. **28**, 3562-3568 (1989).

Graminski, G.F., Zhang, P., Sesay, M.A., Ammon, H.L. & Armstrong, R.N.; Biochem. **28**, 6252-6258 (1989).

Gustafson; Epistat: Student's T-test (two tailed) computer program, version 3.2, Round Rock, Texas, USA (1985).

Guthenberg, C. & Mannervik, B.; Biochem. Biophys. Res. Commun. **86**, 1304-1310 (1979).

Guthenberg, C. & Mannervik, B.; Biochim. Biophys. Acta **661**, 255 (1981).

Guthenberg, C., Åkerfeldt, K. & Mannervik, B.; Acta Chem. Scand. **B33**, 595 (1979).

Guthenberg, C., Ålin, P. & Mannervik, B.; Meth. Enzymol. **113**, 507-510 (1985).

Guthenberg, C., Ålin, P., Åstrand, I.-M., Yalcin, S. & Mannervik, B.; in "Extrahepatic Drug Metabolism and Chemical Carcinogenesis", 171-176, eds. Rydstrom, J., Montelius, J. & Bengtsson, M., Elsevier, Amsterdam (1986).

Habig, W.H. & Jakoby, W.B.; Meth. Enzymol. **77**, 398-405 (1981).

Habig, W.H., Pabst, M.J. & Jakoby, W.B.; Arch. Biochem. Biophys. **175**, 710-716 (1976).

Habig, W.H., Pabst, M.J. & Jakoby, W.B.; J. Biol. Chem. **249**, 7130-7139 (1974).

Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M. & Jakoby, W.B.; Proc. Natl. Acad. Sci. USA **71**, 3879-3882 (1974).

Habig, W.H.; in "Radioprotectors and Anticarcinogens", 169-190, eds. Nygaard, O.F. & Simic, M.G., Academic Press, New York (1983).

Hames, B.D.; in "Gel Electrophoresis of Proteins: A Practical Approach.", 1-86, eds. Hames, B.D. & Rickwood, D., IRL Press Limited, London, U.K. (1981).

Hanson, K.R. & Rose, I.A.; Acc. Chem. Res. **8**, 1-10 (1975).

Harbury, H.A. & Loach, P.A.; J. Biol. Chem. **235**, 3640-3645 (1960).

Harisch, G. & Meyer, W.; Res. Commun. Chem. Path. Pharmacol. **47**, 399-411 (1985).

Hatano, H., Nomura, F., Ohnishi, K., Iijima, T., Hayasaka, A., Iida, S., Koen, H. & Okuda, K.; Hepatology **5**, 241-244 (1985).

Hayakawa, T., Myokei, Y., Yagi, H. & Jerina, D.M.; J. Biochem. **82**, 407-415 (1977).

Hayakawa, T., Udenfriend, S., Yagi, H. & Jerina, D.M.; Arch. Biochem. Biophys. **162**, 438-451 (1975).

Hayes, J.D. & Clarkson, H.D.; Biochem. J. **207**, 459-470 (1982).

Hayes, J.D. & Mantle, T.J.; Biochem. J. **233**, 407-415 (1986).

Hayes, J.D., Strange, R.C. & Percy-Robb, I.W.; Biochem. J. **197**, 491-502 (1981).

Hayes, J.D.; Biochem. J. **213**, 625-633 (1983).

Hazelton, G.A. & Lang, C.A.; Biochem. J. **210**, 281-287 (1983).

Hill, D.L., Shih, T.-W., Johnston, T.P. & Struck, R.F.; Cancer Res. **38**, 2438-2442 (1978).

Hill, D.L., Shih, T.-W., Johnston, T.P. & Struck, R.F.; Cancer Res. **38**, 2438-2442 (1978).

Hoesch, R.M. & Boyer, T.D.; Biochem. J. **251**, 81-88 (1988).

Hong, Y., Li, C.-H., Burgess, J.R., Chang, M., Salem, A., Srikumar, K. & Reddy, C.C.; J. Biol. Chem. **264**, 13793-13800 (1989).

Hunaiti, A.A. & Sarhan, M.A.A.; Int. J. Biochem. **19**, 71-77 (1987).

Inman, W.H.W. & Mushin, W.W.; Br. Med. J. **1**, 5-10 (1974).

Inskeep, P.B. & Guengerich, F.P.; Carcinogenesis **5**, 805-808 (1984).

Ivanetich, K.M. & Goold, R.D.; Biochim. Biophys. Acta **998**, 7-13 (1989).

Ivanetich, K.M., Goold, R.D. & Sikakana, C.N.T.; Biochem. Pharmacol. **39**, 1999-2004 (1990).

Ivanetich, K.M., Ziman, M.R., Mennie, R.E.M., Eidne, K.A., Corrigall, A. & Kirsch, R.E.; Res. Commun. Chem. Path. Pharmacol. **45**, 233-242 (1984).

Jaffe, J.J. & Lambert, R.A.; Mol. Biochem. Parisitol. **20**, 199-206 (1986).

Jakobson, I., Askelöf, P., Warholm, M. & Mannervik, B.; Eur. J. Biochem. **77**, 253-262 (1977).

Jakobson, I., Warholm, M. & Mannervik, B.; Biochem. J. **177**, 861-868 (1979a).

Jakobson, I., Warholm, M. & Mannervik, B.; J. Biol. Chem. **254**, 7085-7089 (1979b).

Jakobsson, I., Warholm, M. & Mannervik, B.; FEBS Letters **102**, 165-168 (1979c).

Jakoby, W.B. & Habig, W.H.; in "Glutathione Transferases: Enzymatic Basis of Detoxification", **2**, 63-95, eds. Jakoby, W.B., Academic Press, New York (1980).

Jakoby, W.B., Habig, W.H., Keen, J.H., Ketley, J.N. & Pabst, M.J.; in "Glutathione: Metabolism and Function", 189-211, eds. Arias, I.M. & Jakoby, W.B., Raven Press, New York, (1976).

Jakoby, W.B., Ketterer, B. & Mannervik, B.; Biochem. Pharmacol. **33**, 2539-2540 (1984).

Jakoby, W.B.; Adv. Enzymol. **46**, 383-414 (1978).

Jakoby, W.B.; in "In Vitro Metabolic Activation in Mutagenesis Testing", 207-212, eds. de Serres, F.J., Fouts, J.R., Bend, J.R. & Philpot, R.M. (1976).

Jakoby, W.B.; Meth. Enzymol. **113**, 495-499 (1985).

Jee, R.C., Sipes, I.G., Gandolfi, A.J. & Brown, B.R.(Jnr.); Toxicol. Appl. Pharmacol. **52**, 267-277 (1980).

Jencks, W.P., in "Catalysis in Chemistry and Enzymology", 4-5, McGraw Hill, New York (1969).

Jensson, H., Ålin, P. & Mannervik, B.; Acta Chem. Scand. **B36**, 205-206 (1982).

Jerina, D.M., Daly, J., Witkop, B., Zaltzman-Nirenberg, P. & Udenfriend, S.; Arch. Biochem. Biophys. **128**, 176-183 (1968).

Johnstone, R.E., Kennell, E.M., Brummund, W. (Jnr.), Shaw, L. M. & Ebersole, R.C.; Clin. Chem. **22**, 217-220 (1976).

Jollow, J.D., Mitchell, J.R., Zampaglione, N. & Gillette, J.R.; Pharmacology **11**, 151-169 (1974).

Jung, G., Breitmaier, E. & Voelter, W.; Eur. J. Biochem. **24**, 438-445 (1972).

Kamisaka, K, Habig, W.H., Ketley, J.N., Arias, I.M. & Jakoby, W.B.; Eur. J. Biochem. **60**, 153-161 (1975).

Kamisaka, K., Listowsky, I. & Arias, I.M.; Annals N. Y. Acad. Sci. **226**, 148-153 (1973).

Kamisaka, K., Listowsky, I., Gatmaitan, Z. & Arias, I.M.; Biochemistry **14**, 2175-2180 (1975).

Kapitulnik, T., Valaes, T., Kaufmann, N.A. & Blondheim, S.H.; Arch. Diseases in Childhood **49**, 886-894 (1974).

Kaplowitz, N., Percy-Robb, I.W. & Javitt, N.B.; J. Exp. Med. **138**, 483-487 (1973).

Kaplowitz, N.; Am. J. Physiol. **239**, G439-G444 (1980).

Karashima, D., Hirokata, Y., Shigematsu, A. & Furukawa, T.; J. Pharmacol. Exp. Ther. **203**, 409-416 (1977).

Kaufmann, N.A., Kapitulnik, J. & Blondheim, S.H.; Clin. Chem. **19**, 1276-1279 (1973).

Keen, J.H. & Jakoby, W.B.; J. Biol. Chem. **253**, 5654-5657 (1978).

Keen, J.H., Habig, W.H. & Jakoby W.B.; J. Biol. Chem. **251**, 6183-6188 (1976).

Keeran, W.S. & Lee, R.F.; Arch. Biochem. Biophys. **255**, 233-243 (1987).

Kenna, J.G., Neuberger, J. & Williams, R.; Biochem. Soc. Trans. **13**, 910-911 (1985).

Ketley, J.N., Habig, W.H. & Jakoby, W.B.; J. Biol. Chem. **250**, 8670-8673 (1975).

Ketterer, B. & Beale, D.; Biochem. J. **122**, 53P (1971).

Ketterer, B., Beale, D. & Meyer, D.; Biochem. Soc. Trans. **10**, 82-84 (1982).

Ketterer, B., Coles, B. & Meyer, D.J.; Environ. Health Perspect. **49**, 59-69 (1983).

Ketterer, B., Neumcke, B. & Lauger, P.; J. Membrane Biol. **5**, 225-245 (1971).

Ketterer, B., Ross-Mansell, P. & Whitehead, J.K.; Biochem. J. **103**, 316-324 (1967).

Ketterer, B., Tipping, E., Beale, D. & Meuwissen, J.; in "Glutathione: Metabolism and Function.", **6**, 243-357, eds. Arias, I. & Jakoby, W., Raven Press, New York (1976).

Ketterer, B., Tipping, E., Beale, D., Meuwissen, J. & Kay, C.M.S.; in "Proceedings XI International Cancer Congress", **2**, 25-29, eds. Bucalossi, P., Veronesi, V. & Cascinelli, N., Florence (1974).

Ketterer, B., Tipping, E., Meuwissen, J. & Beale, D.; Biochem. Soc. Trans. **3**, 626-630 (1975).

Ketterer, B.; Drug Metab. Rev. **13**, 161-187 (1982).

Ketterer, B.; Xenobiotica **16**, 957-973 (1986).

Kitchin, K.T. & Brown, J.L.; Biochem. Biophys. Res. Commun. **141**, 723-727 (1986).

Kluwe, W.M., McNish, R., Smithson, K. & Hook, J.B.; Biochem. Pharmacol. **30**, 2265-2271 (1981).

Knights, K.M., Gourlay, G.K., Gibson, R.A. & Cousins, M.J.; Pharmacol. & Toxicol. **63**, 327-332 (1988).

Knights, K.M., Gourley, G.K. & Cousins, M.J.; Biochem. Pharmacol. **36**, 897-906 (1987).

Koga, N., Inskeep, P.B., Harris, T.M. & Guengerich, F.P.; Biochemistry **25**, 2192-2198 (1986).

Kostyniak, P.J. & Soiefer, A.I.; Toxicol. Lett. **22**, 217-222 (1984).

Kozak, E.M. & Tate, S.S.; J. Biol. Chem. **252**, 6322-6327 (1982).

Laemmli, U.K.; Nature **227**, 680-685 (1970).

Lawrence, R.A. & Burk, R.F.; Biochem. Biophys. Res. Commun. **71**, 952-958 (1976).

Ledda-Columbano, G.M., Columbano, A., Coni, P., Curto, M., Faa, G. & Pani, P.; Toxicol. Letters **37**, 85-90 (1987).

Levi, A.J., Gatmaitan, Z. & Arias, I.M.; J. Clin. Invest. **48**, 2156-2167 (1969).

Levi, A.J., Gatmaitan, Z. & Arias, I.M.; N. Engl. J. Med. **283**, 1136-1139 (1970).

Levine, R.J., Reyes, H., Levi, A.J., Gatmaitan, Z. & Arias, I.M.; Nature New Biol. **231**, 277-279 (1971).

Li, C.-H., Srikumar, K., Burgess, J.R., Hong, Y., Hildenbrandt, G., Rao, M.K., Reddanna, P., Li, N. & Tu, C.-P.D. & Reddy, C.C.; ICSU Short Reports, **6**, 662-663, eds. Kon, O.L. *et al.*, Cambridge University Press, Cambridge, U.K.. (1986).

Lichter, M., Fleischner, G., Kirsch, R., Levi, A.J., Kamisaka, K. & Arias, I.M.; Am. J. Physiol. **230**, 1113-1120 (1976).

Lind, R.C., Gandolfi, A.J. & Hall, P. de la M.; Anesthesiology **70**, 649-653 (1989).

Litwack, G., Ketterer, B. & Arias, I.M.; Nature **234**, 466-467 (1971).

Livesey, J.C., Anders, M.W., Langvardt, P.W., Putzig, C.L. & Reitz, R.H.; Drug Metab. Distrib. **10**, 201-204 (1982).

Loew, G. & Goldblum, A.; J. Am. Chem. Soc. **102**, 3657-3659 (1980).

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J.; J. Biol. Chem. **192**, 265-275 (1951).

Lunam, C.A., Cousins, M.J. & Hall, P. de la M.; J. Pharmacol. Exp. Ther. **232**, 802-809 (1985).

Malhotra, S.K. & Ringold, H.J.; J. Am. Chem. Soc. **87**, 3228-3236 (1965).

Mangold, J.B. & Abdel-Monem, M.M.; Biochem. Biophys. Res. Commun. **96**, 333-340 (1980).

Mangold, J.B. & Abdel-Monem, M.M.; J. Med. Chem. **26**, 66-71 (1983).

Mannervik, B. & Askelöf, P.; FEBS Letters **56**, 218-221 (1975).

Mannervik, B. & Danielson, U.H.; CRC Crit. Rev. Biochem. **23**, 283-337 (1988).

Mannervik, B. & Guthenberg, C.; Meth. Enzymol. **77**, 231-235 (1981).

Mannervik, B. & Jensson, H.; J. Biol. Chem. **257**, 9909-9912 (1982).

Mannervik, B., Ålin, P., Guthenberg, C., Jensson, H., Tahir, M.K., Warholm, M. & Jörnvall, H.; Proc. Natl. Acad. Sci. USA **82**, 7202-7206 (1985).

Mannervik, B., Guthenberg, C., Jensson, H., Warholm, M. & Ålin, P.; in "Functions of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects.", 75-88, eds. Larsson, A., Orrenius, S., Holmgren, A. & Mannervik, B., Raven Press, New York (1983).

Mannervik, B., Jensson, H., Ålin, P., Öring, L. & Hammarström, S.; FEBS Letters **174**, 289-293 (1984).

Mannervik, B.; Adv. Enzymol. **57**, 357-417 (1985).

Mansuy, D., Nastainczyk, W. & Ullrich, V.; Naunyn-Schmiedeberg's Arch. Pharmacol. **285**, 315-324 (1974).

Mapes, J.P.; Biochem. J. **162**, 47-50 (1977).

Marchand, D.H. & Abdel-Monem, M.M.; Biochem. Biophys. Res. Commun. **128**, 360-367 (1985).

Marcus, C.J., Habig, W.H. & Jakoby, W.B.; Arch. Biochem. Biophys. **188**, 287-293 (1978).

Marsh, J.A., Lucas, S.A., Harrison, G.G. & Ivanetich, K.M.; S.A. Med. J. **55**, 871-877 (1979).

Maruyama, H. & Listowsky, I.; J. Biol. Chem. **259**, 12449-12455 (1984).

Maruyama, H., Arias, I.M. & Listowsky, I.; J. Biol. Chem. **259**, 12444-12448 (1984).

McCarthy, L.P., Malek, R.S. & Larsen, E.R.; Anesthesiology **51**, 106-110 (1979).

McCaughey, W.; Br. J. Anaesth. **44**, 918 (1972).

McLain, G.E., Sipes, I.G., Brown, B.R.; Anesthesiology **51**, 321-326 (1979).

Meister, A. & Anderson, M.E.; Ann. Rev. Biochem. **52**, 711-760 (1983).

Meister, A.; J. Biol. Chem. **263**, 17205-17208 (1988).

Meyer, D.J. & Ketterer, B.; FEBS Letters **150**, 499-502 (1982).

Meyer, D.J., Beale, D., Tan, K.H., Coles, B. & Ketterer, B.; FEBS Letters **184**, 139-143 (1985).

Meyer, D.J., Lalor, E., Coles, B., Kispert, A., Ålin, P., Mannervik, B. & Ketterer, B.; Biochem. J. **260**, 785-788 (1989).

Mieli-Vergani, G., Vergani, D., Tredger, J.M., Eddleston, A.L.W.F., Davis, M. & Williams, R.; J. Clin. Lab. Immunol. **4**, 49-51 (1980).

Mitchell, J.R., Jollow, D.J., Potter, W.Z., Gillette, J.R. & Brodie, B.B.; J. Pharmacol. Exp. Ther. **187**, 211-217 (1973).

Mitchell, J.R., Thorgeirsson, S.S., Potter, W.Z., Jollow, D.J. & Keiser, H.; Clin. Pharmacol. Therap. **16**, 676-684 (1974).

Miyaura, S. & Isono, H.; Chem. Pharm. Bull. **34**, 194-205 (1986).

Mönig, J., Krischer, K. & Asmus, K.-D.; Chem.-Biol. Interact. **45**, 43-52 (1983).

Morey, K.S. & Litwack, G.; Biochemistry **8**, 4813-4821 (1969).

Morgenstern, R. & DePierre, J.W.; Eur. J. Biochem. **134**, 591-597 (1983).

Morgenstern, R., DePierre, J.W. & Ernster, L.; Acta Chem. Scand. **B34**, 229-230 (1980).

Morgenstern, R., DePierre, J.W. & Ernster, L.; Biochem. Biophys. Res. Commun. **87**, 657-663 (1979).

Morgenstern, R., DePierre, J.W. & Jörnvall, H.; J. Biol. Chem. **260**, 13976-13983 (1985).

Morgenstern, R., Guthenberg, C., Mannervik, B., DePierre, J.W. & Ernster, L.; Cancer Res. **42**, 4215-4221 (1982).

Morgenstern, R., Lundquist, G., Andersson, G., Balk, L. & DePierre, J.W.; Biochem. Pharmacol. **33**, 3609-3614 (1984).

Morgenstern, R., Meijer, J., DePierre, J.W. & Ernster, L.; Eur. J. Biochem. **104**, 167-174 (1980).

Moslen, M.T. & Reynolds, E.S.; Res. Commun. Chem. Path. Pharmacol. **47**, 59-72 (1985).

Mozer, T.J., Tiemeier, D.C. & Jaworski, E.J.; Biochemistry **22**, 1068-1072 (1983).

Mukhtar, H. & Bend, J.R.; Life Sciences **21**, 1277-1286 (1977).

Mullin, C.A., Croft, B.A., Strickler, K., Matsumura, F. & Miller, J.R.; Science **217**, 1270-1272 (1982).

Nachtomi, E. & Sarma, D.S.R.; Biochem. Pharmacol. **26**, 1941-1945 (1977).

Nachtomi, E., Alumot, E. & Bondi, A.; Israel J. Chem. **4**, 239-246 (1966).

Nachtomi, E.; Biochem. Pharmacol. **19**, 2853-2860 (1970).

Nastainczyk, W., Ullrich, V. & Sies, H.; Biochem. Pharmacol. **27**, 387-392 (1978).

National Halothane Study; eds. Bunker, J.P., Forrest, W.H., Mosteller, F. & Vandam, L.D., U.S.A. Goverment Printing Office, Washington D.C. (1969).

Neuberger, J. & Kenna, J.G.; Clin. Sci. **72**, 263-270 (1987).

Neuberger, J. & Williams, R.; Br. Med. J. **289**, 1136-1139 (1984).

Nielsen, P. & Rasmussen, F.; Life Sci. **17**, 1495-1512 (1977).

Nimmo, I.A., Clapp, J.B. & Strange, R.C.; Comp. Biochem. Physiol. **63B**, 423-427 (1979).

Odell, G. & Gourley, G.R.; in "Textbook of Gastroenterology and Nutrition in Infancy.", eds. Lebenthal, E., 949-967, Raven Press, New York (1989).

Ogorochi, T., Ujihara, M. & Narumiya, S.; J. Neurochem. **48**, 900-909 (1987).

Ohkawa, H., Ohkawa, R., Yamamoto, I. & Casida, J.E.; Pestic. Biochem. Physiol. **2**, 95 (1972).

Oleaga, A., Gonzalez, J. & Esteller, A.; Comp. Biochem. Physiol. **87**, 13-20 (1987).

Ong, L.K. & Clark, A.G.; Biochem. Pharmacol. **35**, 651-654 (1986).

Ostlund Farrants, A.-K., Meyer, D.J., Coles, B., Southan, C., Aitken, A., Johnson, P.J. & Ketterer, B.; Biochem. J. **245**, 423-428 (1987).

Overbaugh, J.M., Lau, E.P., Marino, V.A. & Fall, R.; Arch. Biochem. Biophys. **261**, 227-234 (1988).

Owen, A.D. & Van der Veen, B.W.; S.A. Med. J. **69**, 807-810 (1986).

Ozawa, N. & Guengerich, F.P.; Proc. Natl. Acad. Sci. USA **80**, 5266-5270 (1983).

Pabst, M.J., Habig, W.H. & Jakoby, W.B.; Biochem. Biophys. Res. Commun. **52**, 1123-1129 (1973).

Pabst, M.J., Habig, W.H., & Jakoby, W.B.; J. Biol. Chem. **249**, 7140-7150 (1974).

Pattinson, N.; Anal. Biochem. **115**, 424-427 (1981).

Pearson, R.G. & Songstad, J., J. Am. Chem. Soc. **89**, 1827-1836 (1967).

Peterson, J., Saleem, M.M.M., Silver, J. & Wilson, M.T.; J. Inorg. Biochem. **19**, 165-178 (1983).

Peterson, J., Saleem, M.M.M., Silver, J., Wilson, M.T., & Morrison, I.E.G.; J. Inorg. Biochem. **19**, 165-178 (1983).

Peterson, J., Silver, J., Wilson, M.T. & Morrison, I.E.G.; J. Inorg. Biochem. **13**, 75-82 (1980).

Plattner, H., Wachter, E. & Gröbner, P.; Histochem. **53**, 223-242 (1977).

Plummer, J.L., Hall, P. de la M., Jenner, M.A. & Cousins, M.J.; Anesthesiology **67**, 355-360 (1987).

Pohl, L.R. & Gillette, J.R.; Drug Metab. Rev. **15**, 1335-1351 (1984).

Polidoro, G., Del Boccio, G., Di Ilio, C., Piccolomini, R., Ravagnan, G. & Federici, G.; Res. Commun. Chem. Path. Pharmacol. **46**, 411-423 (1984).

Polidoro, G., Di Illio, C., Del Boccio, G., Zulli, P. & Federici, G.; Biochem. Pharmacol. **29**, 1677-1680 (1980).

Potter, W.Z., Thorgeirsson, S.S., Jollow, D.J. & Mitchell, J.R.; Pharmacology **12**, 129-143 (1974).

Principato, G.B., Danielson, U.H. & Mannervik, B.; FEBS Letters **231**, 155-158 (1988).

Prohaska, J.R. & Ganther, H.F.; Biochem Biophys. Res. Commun. **76**, 437-445 (1977).

Radulovic, L.L. & Kulkarni, A.P.; Biochem. Biophys. Res. Commun. **128**, 75-81 (1985).

Radulovic, L.L. & Kulkarni, A.P.; Biochem. J. **239**, 53-57 (1986).

Ramage, P.I.N. & Nimmo, I.A.; Biochem. J. **211**, 523-526 (1983).

Rannug, U.; Mut. Res. **76**, 269-295 (1980).

Ratcliffe, J.M., Schrader, S.M., Steenland, K., Clapp, D.E., Turner, T. & Hornung, R.W.; Br. J. Ind. Med. **44**, 317-326 (1987).

Raventos, J.; Br. J. Pharmacol. **11**, 394-410 (1956).

Reddy, C.C., Burgess, J.R. Gong, Z.-Z., Massaro, E.J. & Tu, C.-P.D.; Arch. Biochem. Biophys. **224**, 87-101 (1983).

Rehder, K., Forbes, J., Alter, H., Hessler, O. & Stier, A.; Anesthesiology **28**, 711-715 (1967).

Reilly, C.S., Wood, A.J.J., Koshakji, R.P. & Wood, M.; Anesthesiology **63**, 70-76 (1985).

Reuben, D.M.E. & Bruice, T.C.; J. Am. Chem. Soc. **98**, 114-121 (1976).

Reyes, H., Levi, A.J., Gatmaitan, Z. & Arias, I.M.; J. Clin. Invest. **50**, 2242-2252 (1971).

Rice, S.A., Maze, M., Smith, C.M., Kosek, J.C. & Mazze, R.I.; Toxicol. Appl. Pharmacol. **87**, 411-419 (1987).

Riechen, J., Blitzer, B.L. & Berk, P.D.; Biochim. Biophys. Acta **640**, 298-312 (1981).

Rietjens, I.M.C.M., Lemmink, H.H., Alink, G.M. & van Bladeren, P.J.; Chem.-Biol. Interact. **62**, 3-14 (1987).

Rosenberg, H. & Pohl, S.; Life Sci. **17**, 431-434 (1975).

Ross J.A.S., Monk, S.J. & Duffy S.W.; Br. J. Anaesth. **56**, 527-532 (1984).

Ross, T.R. & Daggy, B.P.; Anesth. Analg. **60**, 306-309 (1981).

Ross, W.T., Daggy, B.P. & Cardell, R.R.; Anesthesiology **51**, 327-333 (1979).

Ruf, H. H., Ahr, H., Nastainczyk, W., Ullrich, V., Mansuy, D., Battioni, J.-P., Montiel-Montoya, R.F. & Trautwein, A.; Biochemistry **23**, 5300-5306 (1984).

Sachdev, K., Cohen, E.N. & Simmon, V.F.; Anesthesiology **53**, 31-39 (1980).

Salemme, F.R., Kraut, J. & Kamen, M.D.; J. Biol. Chem.; **248**, 7701-7716 (1973).

Salemme, F.R.; Ann. Rev. Biochem. **46**, 299-329 (1977).

Sasame, H.A., Liberato, D.J. & Gillette, J.R.; Drug Metab. Dispos. **15**, 349-355 (1987).

Satoh, H., Fukuda, Y., Anderson, D.K., Ferrans, V.J., Gillette, J.R. & Pohl, L.R.; J. Pharmacol. Exp. Ther. **233**, 857-862 (1985).

Satoh, H., Gillette, J.R., Takemura, T., Ferrans, V.J., Jelenich, S.E., Kenna, J.G., Neuberger, J. & Pohl, L.R.; in "Biological Reactive Intermediates III.", 657-673, eds. Koosis, J.J., Jollow, D.J., Witmer, C.M., Nelson, J.O. & Snyder, R., Plenum Publishing Corporation, New York (1986).

Schieble, T.M., Costa, A.K., Heffel, D.F. & Trudell, J.R.; Anesthesiology **68**, 485-494 (1988).

Schramm, V.L., McCluskey, R., Emig, F.A. & Litwack, G.; J. Biol. Chem. **259**, 714-722 (1984).

Scully, N.C. & Mantle, T.J.; Biochem. Soc. Trans. **8**, 451-452 (1980).

Segel, I.M.; in "Enzyme Kinetics. Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems.", John Wiley & Sons, New York (1975).

Seidegard, J., DePierre , J.W., Birberg, W., Pilotti, A. & Pero, R.W.; Biochem. Pharmacol. **33**, 3053-3058 (1984).

Senjo, M. & Ishibashi, T.; Biomed. Res. **7**, 19-26 (1986).

Shack, J. & Clark, W.M.; J. Biol. Chem. **171**, 143-187 (1947).

Sheehan, D. & Mantle, T.J.; Biochem. J. **218**, 893-897 (1984).

Shishido, T.; Agric. Biol. Chem. **45**, 2951 (1981).

Siadat-Pajouh, M., Hubbard, A.K., Roth, T.P. & Gandolfi, A.J.; Anesth. Analg. **66**, 1209-1214 (1987).

Simons, P.C. & Vander Jagt, D.L.; Anal. Biochem. **82**, 334-341 (1977).

Simons, P.C. & Vander Jagt, D.L.; J. Biol. Chem. **255**, 4740-4744 (1980).

Simplicio, J. & Schwenzer, K.; Biochem. **12**, 1923-1929 (1973).

Singh, S.V., Ansari, G.A.S. & Awasthi, Y.C.; J. Chrom. **361**, 337-345 (1986).

Singh, S.V., Dao, D.D., Srivastava, S.K. & Awasthi, Y.C.; Curr. Eye Res. **3**, 1273-1280 (1984).

Sipes, I.G., Gandolfi, A.J., Pohl, L.R., Krishna, G. & Brown, B.R.; J. Pharmacol. Exp. Ther. **214**, 716-720 (1980).

Smith, A., Nuiry, I.& Awasthi, Y.C.; Biochem. J. **229**, 823-831 (1985).

Smith, A.C., Roberts, S.M., James, R.C., Berman, L.M. & Harbison, R.D.; Xenobiotica **18**, 991-1001 (1988).

Smith, G.J., Ohl, V.S. & Litwack, G.; Cancer Res. **37**, 8-14 (1977).

Smith, S.B., Richards, J.W. & Benisek, W.F.; J. Biol. Chem. **255**, 2685-2689 (1980).

Sodeman, W.A. & Sodeman, T.M.; in "Sodeman's Pathologic Physiology, Mechanisms of Disease.", 887-896, W.B. Saunders Company, London (1985).

Söderström, M., Hammarström, S. & Mannervik, B.; Biochem. J. **250**, 713-718 (1988).

Söderström, M., Mannervik, B., Örning, L. & Hammarström S.; Biochem. Biophys. Res. Commun. **128**, 265-270 (1985).

Soma, Y., Satoh, K. & Sato, K.; Biochim. Biophys. Acta **869**, 247-258 (1986).

Spector, L.B.; in "Covalent Catalysis by Enzymes.", 196-197, Springer-Verlag, New York (1982).

Stenger, R.J. & Johnson, E.A.; Proc. Soc. Exp. Biol. Med. **140**, 1319-1324 (1972).

Stephen, C.R., Grosskreutz, J.H., Lawrence, J.H.A., Fabian, L.W., Bourgeois-Gavardin, M. & Coughlin, J.; Can. Anaesth. Soc. J. **4**, 246-258 (1957).

Stier, A., Alter, H., Hessler, O. & Rehder, K.; Anesth. Analg. **43**, 723-728 (1964).

Stockman, P.K., Beckett, G.J. & Hayes, J.D.; Biochem. J. **227**, 457-465 (1985).

Stockman, P.K., McLellan, L.I. & Hayes, J.D.; Biochem. J. **244**, 55-62 (1987).

Stolz, A., Takikawa, H., Ookhtens, M. & Kaplowitz, N.; Ann. Rev. Physiol. **51**, 161-176 (1989).

Stremmel, W., Gerber, M.A., Glezerov, V., Thung, S.N., Kochwa, S. & Berk, P.D.; J. Clin. Invest. **71**, 1796-1805 (1983).

Sugiyama, Y., Sugimoto, M., Stolz, A.W. & Kaplowitz, N.; Biochem. Pharmacol. **33**, 3511-3513 (1984).

Sugiyama, Y., Yamada, T. & Kaplowitz, N.; Biochem. J. **199**, 749-756 (1981).

Sundheimer, D.W., White, R.D., Brendel, K. & Sipes, I.G.; Carcinogenesis **3**, 1129-1133 (1982).

Suzuki, T., Coggan, M., Shaw, D.C. *et al*; Ann. Hum. Genet. **51**, 95-106 (1987).

Tahir, M.K., Guthenberg, C. & Mannervik, B.; FEBS Letters **181**, 249-252 (1985).

Talalay, P. & Wang, V.S.; Biochim. Biophys. Acta **18**, 300-301 (1955).

Tamaki, H., Kumagai, H. & Tochikura, T.; J. Bacteriol. **171**, 1173-1177 (1989).

Tan, K.H., Meyer, D.J., Belin, J. & Ketterer, B.; Biochem. J. **220**, 243-252 (1984).

Tateishi, M. & Shimizu, H.; in "Enzymatic Basis of Detoxication", **2**, 120-130, eds. W.B. Jakoby, Academic Press, New York (1980).

Tateoka, N., Tsuchida, S., Soma, Y. & Sato, K.; Clin. Chem. Acta **166**, 207-218 (1987).

Thorne, C.J.R.; Techniques in Protein and Enzyme Biochemistry **B104**, 1-18 (1978).

Tietze, F.; Anal. Biochem. **27**, 502-522 (1969).

Tipping, E. & Ketterer, B.; Biochem. J. **195**, 441-452 (1981).

Tipping, E., Ketterer, B. & Koskelo, P.; Biochem. J. **169**, 509-516 (1978).

Tipping, E., Ketterer, B., Christodoulides, L. & Enderby, G.; Biochem. J. **157**, 211-216 (1976).

Tipping, E., Ketterer, B., Christodoulides, L. & Enderby, G.; Eur. J. Biochem. **67**, 583-590 (1976).

Touloukian, J. & Kaplowitz, N.; Semin. Liver Dis. **1**, 134-142 (1981).

Trowell, J., Peto, R. & Crampton Smith, A.; Lancet **1**, 821-824 (1975).

Tsou, C.L.; Biochem. J. **49**, 362-367 (1951).

Tsuchiya, M., Okimasu, E., Ueda, W., Hirakawa, M. & Utsumi, K.; FEBS Letters **242**, 101-105 (1988).

Tu, C.-P.D., Chang, M. & Reddy, C.C.; Biochem. Biophys. Res. Commun. **123**, 981-988 (1984).

Uehleke, H., Hellmer, K.H. & Tabarelli-Poplawski, S.; Naunyn-Schmiedeberg's Arch. Pharmacol. **279**, 39-52 (1973).

Urry, D.W. & Pettegrew, J.W.; J. Am. Chem. Soc. **89**, 5276-5283 (1967).

Urry, D.W.; J. Am. Chem. Soc. **89**, 4190-4196 (1967).

USEPA (U.S. Environmental Protection Agency); Federal Register **42**, 63134-63148 (1977).

van Bladeren, P.J., Breimer, D.D., Rotteveel-Smijs, G.M.T., de Knijff, P., Mohn, G.R., van Meeteren-Walchli, B., Buijs, W. & van der Gen, A.; Carcinogenesis **2**, 499-505 (1981).

van Bladeren, P.J., Breimer, D.D., Rotteveel-Smijs, G.M.T., Hoogeterp, J.J., Mohn, G.R., de Groot, A., van Zeeland, A.A. & van der Gen, A.; Adv. Exp. Med. Biol. **136**, 809-820 (1981).

Van Dyke, R.A. & Gandolfi, A.J.; Drug Metab. Dispos. **4**, 40-44 (1976).

Vandenberghe, Y., Glaise, D., Meyer, D.J., Guillouzo, A. & Ketterer, B.; Biochem. Pharmacol. **37**, 2482-2485 (1988).

Vander Jagt, D.L., Dean, V.L., Wilson, S.P. & Royer, R.E.; J. Biol. Chem. **258**, 5689-5694 (1983).

Vander Jagt, D.L., Hunsaker, L.A., Garcia, K.B. & Royer, R.E.; J. Biol. Chem. **260**, 11603-11610 (1985).

Vander Jagt, D.L., Wilson, S.P. & Heidrich, J.E.; FEBS Letters **136**, 319-321 (1981).

Vander Jagt, D.L., Wilson, S.P., Dean, V.L. & Simons, P.C.; J. Biol. Chem. **257**, 1997-2001 (1982).

van Bladeren, P.J., Breimer, D.D., Rotteveel-Smijs, G.M.T. & Mohn, G.R.; Mutation Res. **74**, 341-346 (1980).

van Bladeren, P.J., Breimer, D.D., Rotteveel-Smijs, G.M.T., De Jong, R.A.W., Buijs, W., van der Gen, A. & Mohn, G.R.; Biochem. Pharmacol. **29**, 2975-2982 (1980).

van Bladeren, P.J., van der Gen, A., Breimer, D.D. & Mohn, G.R.; Biochem. Pharmacol. **28**, 2521-2524 (1979).

van Bladeren, P.J.; Trends Pharmacol. Sci. **9**, 295-299 (1988).

Van Dyke, R.A. & Gandolfi, A.J.; Drug Metab. Dispos. **2**, 469-476 (1974).

Van Dyke, R.A., Baker, M.T., Jansson, I. & Schenkman, J.; Biochem. Pharmacol. **37**, 2357-2361 (1988).

Van Iersel, A.A.J., De Boer, A.J., Van Holsteijn, C.W.M. & Blaauboer, B.J.; Fed. Chem. Toxic. **24**, 569-570 (1986).

Van Iersel, A.A.J., De Boer, A.J., Van Holsteijn, C.W.M. & Blaauboer, B.J.; Toxic. *in vitro* **2**, 75-81 (1988).

Vergani, D., Tsantoulas, D., Eddleston, A.L.W.F., Davis, M. & Williams, R.; Lancet **2**, 801-803 (1978).

Vessey, D.A. & Boyer, T.D.; Toxicol. Appl. Pharmacol. **73**, 492-499 (1984).

Viger, A., Coustal, S. & Marquet, A.; J. Am. Chem. Soc. **103**, 451-458 (1981).

Vince, R., Daluge, S. & Wadd, W.B.; J. Med. Chem. **14**, 402-404 (1971).

Vos, R.M.E., Rietjens, M.C.M., Alink, G.M. & van Bladeren, P.J.; Eur. J. Drug Metab. Pharmacokinet. **12**, 275-277 (1987).

Walton, B., Simpson, B.R., Strunin, L., Doniach, D., Perrin, J. & Appleyard, A.J.; Br. Med. J. **1**, 1171-1176 (1976).

Wang, S.-F., Kawahara, F.S. & Talalay, P.; J. Biol. Chem. **238**, 576-585 (1963).

Warholm, M., Guthenberg, C. & Mannervik, B.; Biochem. **22**, 3610-3617 (1983).

Warholm, M., Guthenberg, C., Mannervik, B., von Bahr, C. & Glaumann, H.; Acta Chem. Scand. **B34**, 607-621 (1980).

Warholm, M., Jensson, H., Tahir, M.K. & Mannervik, B.; Biochemistry **25**, 4119-4125 (1986).

Weisburger, E.K.; Am. Chem. Soc. Meeting, 1-23 (1985).

Wendel, A. & Cikryt, P.; Res. Commun. Chem. Path. Pharmacol. **33**, 463-473 (1981).

Wendel, A.; Meth. Enzymol. **77**, 325-333 (1981).

White, R.D., Petry, T.W. & Sipes, I.G.; Chem. Biol. Interact. **49**, 225-233 (1984).

Wiener, H.; Eur. J. Biochem. **157**, 351-363 (1986).

Wiersma, D.A., Schnellmann, R.G. & Sipes, I.G.; J. Biochem. Toxicol. **1**, 1-12 (1986).

Wilchek, M.; Anal. Biochem. **49**, 572-575 (1972).

Williams, R.T.; in "Detoxification Mechanisms", 237-277, John Wiley & Sons, New York (1959).

Williamson, G. & Beverley, M.C.; Biochem. Soc. Trans. **15**, 1103-1104 (1987).

Williamson, G. & Beverley, M.C.; J. Cereal Sci. **8**, 155-164 (1988).

Williamson, G., Ball, S.K.M. & Chan, H.W.S.; Biochem. Soc. Trans. **14**, 1278-1279 (1986).

Wilson, M.T., Ranson, R.J., Masiakowski, P., Czarnecka, E. & Brunori, M.; Eur. J. Biochem. **77**, 193-199 (1977).

Wolkoff, A.W. & Chung, C.T.; J. Clin. Invest. **65**, 1152-1161 (1980).

Wolkoff, A.W., Goresky, C.A., Sellin, J., Gatmaitan, Z. & Arias, I.M.; Am. J. Physiol. **236**, E638-E648 (1979).

Wolkoff, A.W., Weisiger, R.A. & Jakoby, W.B.; in "Progress in Liver Diseases", **6**, 213-224, eds. Popper, H. & Schaffner, F., Irvine & Stratton Inc., New York (1979).

Wong, L.C.K., Winston, J.M., Hong, C.B. & Plotnick, H.; Toxicol. Appl. Pharmacol. **63**, 155-165 (1982).

Woolley, P.V., Hunter, M.J. & Arias, I.M.; Biochim. Biophys. Acta **446**, 115-123 (1976).

Working, P.K., Smith-Oliver, T., White, R.D. & Butterworth, B.E.; Carcinogenesis **7**, 476-472 (1986).

Wright, R., Chisholm, M., Lloyd, B., Edwards, J.C., Eade, O.E., Hawksley, M., Moles, T.M. & Gardner, M.; Lancet **1**, 821-823 (1975).

Yamada, T. & Kaplowitz, N.; Biochem. Pharmacol. **29**, 1205-1208 (1980).

Yamada, T. & Kaplowitz, N.; J. Biol. Chem. **255**, 3508-3513 (1980).

Yoshimoto, T., Soberman, R.J., Lewis, R.A. & Austen, K.F.; Proc. Natl. Acad. Sci. USA **82**, 8399-8403 (1985).

Younes, M., Heger, B., Wilhelm, K-P, Siegers, C.-P.; Pharmacol. Toxicol. **63**, 52-56 (1988).

Younes, M., Schlichting, R. & Siegers, C.-P.; Pharmacol. Res. Commun. **12**, 921-930 (1980).

Young, P.R. & Briedis, A.V.; Biochem. J. **257**, 541-548 (1989).

Zampaglione, N., Jollow, D.J., Mitchell, J.R., Stripp, B., Hamrick, M. & Gillette, J.R.; J. Pharmacol. Exp. Ther. **187**, 218-227 (1973).

Zoetemelk, C.E.M., Mohn, G.R., Van Der Gen, A. & Breimer, D.D.; Biochem. Pharmacol. **36**, 1829-1836 (1987).