GLUTATHIONE S-TRANSFERASES AND ARACHIDONIC ACID CASCADE: EFFECTS OF DIETARY VITAMIN E AND SELENIUM IN FEMALE ALBINO RATS

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY
ANURADHA DESAI

SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD 500 046
INDIA

1997

Enrolment No. PL 5989

To my parents

STATEMENT

1 hereby state that the work presented in this thesis entitled **"Glutathione S-transferases and arachidonic acid cascade: Effects of dietary vitamin E and Selenium in female albino rats"** has been carried out by me under the supervision of Dr. P. Reddanna and that this has not been submitted for any degree or diploma of any other University earlier.

(Dr. P. Reddanna)
Research supervisor

Dr. P. Reddanna
READER
School of Life Sciences
Univ of Hyderabad
HYDERABAD - 500 134

(D. Anuradha)
Research student

CERTIFICATE

This is to certify that Ms. **D. Anuradha,** has carried out the research embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph.D. ordinance of this University. I recommend her thesis entitled **"Glutathione S-transferases and arachidonic acid cascade: Effects of dietary vitamin E and Selenium in female albino rats"** for submission for the degree of Doctor of Philosophy of this University.

Dr. P.Reddanna
Supervisor
Dr. P. Reddanna
READER
School of Life Sciences
Univ of Hyderabad
HYDERABAD - 500 134.

Prof. P.R.K.Reddy
Head,
Deptt. of Animal Sciences
HEAD
DEPTT. OF ANIMAL SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD - 500 046

Prof. A.R.Reddy
DEAN,
School of Life Sciences
Dean, School of Life Sciences
University of Hyderabad,
Hyderabad-500 134. (India)

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA	arachidonic acid
Ab	antibody
ARE	antioxidant responsive element
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BHT	butylated hydroxytoulene
CDNB	1-Chloro-2,4-dinitrobenzene
CHP	cumene hydroperoxide
CM-cellulose	carboxy methyl cellulose
COX	cyclooxygenase
DAN	diaminonapthalene
DCNB	1,2-dichloro-4-nitrobenzene
DE 52	diethyl aminoethyl cellulose
FAHP	fatty acid hydroperoxide
GCS	γ-glutamylcysteine synthetase
GGT	γ-glutamyl transpeptidase
GSH	glutathione reduced
GSSG	glutathione oxidized
GST	glutathione S-transferase
HETE	hydroxyeicosatetraenoic acid
HODE	hydroxyoctadecadienoic acid
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
HPODE	hydroperoxyoctadecadienoic acid
LOX	lipoxygenase
LT	leukotriene
LX	lipoxin
MDA	malondialdehyde
NBT	nitroblue tetrazolium
Non-Se-GSH Px	non-selenium-glutathione peroxidase
PAH	poly aromatic hydrocarbon
PG	prostaglandin
PGI	prostacyclin
PLA_2	phospholipase A_2
PUFA	polyunsaturated ferry acid
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

Se	selenium
Se-GSH Px	selenium-glutathione peroxidase
SOD	superoxide dismutase
TBA	thiobarbituric acid
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TX or Tx	thromboxane
vit.E	vitamin E
XRE	xenobiotic responsive element
g	gram
hr	hour
L	liter
u	micro
M	molar
min	minute
nm	nanometer
s	second

A CKNO WLEDGEMENTS

I would like to place on record my deep sense of gratitude to Dr. P. Reddanna, for his inspiring and expert guidance in carrying out this study. I feel privileged for having stimulating discussion with him and for his critical suggestions. I am very much indebted to him for the constant encouragement and affection shown during the years of my association with him.

I would like to thank Prof. A. R. Reddy, Dean, School of Life Sciences and Prof. P.R.K. Reddy, Head, Department of Animal Sciences for providing the necessary facilities for this study.

I wish to thank Prof. Ch. R. K. Murthy, Dr. Mohan C. Vemuri and Dr. Aparna Dutta Gupta for extending their lab facilities.

I would like to thank Dr. Srinivas Reddy , Mr. Muralidhar Reddy, Mr. Ramakrishna, Dr. Narasa Reddy, Dr. Kiranmai, Mrs. Usha, Mrs. Uma, Mr. Madhava Reddy, Ms. Neeraja, Mr. Bhagavan, Mr. Raheem and former colleagues for the good times we spent together in the lab.

I am very thankful to Dr. Hildenbrandt of Penn State University for critically reading the manuscript.

I thank Mr. Murali, Dr. Rama Devi, Mr. Kantha Rao, Mr. Thaha, Dr .Prabhavathi, Dr. Rukmini Devi, Dr. Prasuna and Ms. Vasanthi for their lively company during my stay in the campus.

I thank Mr. Nagesh, Mr.Sivaram and Mr. Sudhakar for their assistance in the lab.

I wish to express my profound gratitude to my grand mother, mother-in-law, parents, Prasad mamayya, sisters, brother, brothers-in-law, Vijayakka, Lalitha, Jayaram anna, Ramana, and Dasarath but for whose patience, moral support, understanding and encouragement, this could not have been successfully completed

I wish to thank my husband Veeru for his friendship, balance sense of humor and lively company which made my stay in this lab a memorable one. His continuous help and co-operation together with his patient endurance has been instrumental in many ways.

Financial assistance from Indian Council of Medical Research is gratefully acknowledged.

Desai

INTRODUCTION

GENERAL INTRODUCTION

1.1.0.0. Glutathione S-transferases

Drug detoxification mechanism is an inherent system of living organisms. Xenobiotics are detoxified and eliminated from the body by either phase I or phase II drug detoxification systems (Fig. 1). Phase I metabolism involves an initial oxidation of the xenobiotics by cytochrome P450 (CYP) monooxygenases. This step is followed by phase II metabolism, which frequently involves conjugation reactions catalyzed by glutathione S-transferases (GSTs), UDP-glucuronosyl transferases, and sulfotransferases. Some of the xenobiotics like diphenols and thiocarbamates directly activate GSTs of phase II detoxification system. The glutathione (GSH) conjugates thus formed are transported out of cells for further metabolism by γ–glutamyl transpeptidase and dipeptidases, that catalyze the sequential removal of the glutamyl and glycyl moieties, respectively. The resulting cysteine S-conjugates can be acetylated by intracellular N-acetyl-transferase to form the corresponding mercapturic acids (N-acetyl-cysteine S-conjugates) which are then released into the circulation and delivered to the kidney for excretion in urine, or they may undergo further metabolism (Fig. 2). Mercapturic acid biosynthesis is generally considered to be an interorgan process, with the liver serving as the major site of glutathione conjugation and kidney being the primary site for conversion of glutathione conjugates to cysteine conjugates (Fig. 3). Thus GSTs comprise a family of phase II drug detoxification enzymes catalyzing the conjugation of GSH with the electrophilic compounds produced from biotransformation of exogenous xenobiotics and also from endogenous substances (Mannervik and Danielson, 1988; Coles and Ketterer, 1990; Tsuchida and Sato, 1992).

1.1.2.0. Classification of GSTs

In mammals cytosolic GSTs are encoded by at least 4 multigene families which include alpha (a), mu (μ), pi (π), and theta (9) class GSTs (Mannervik

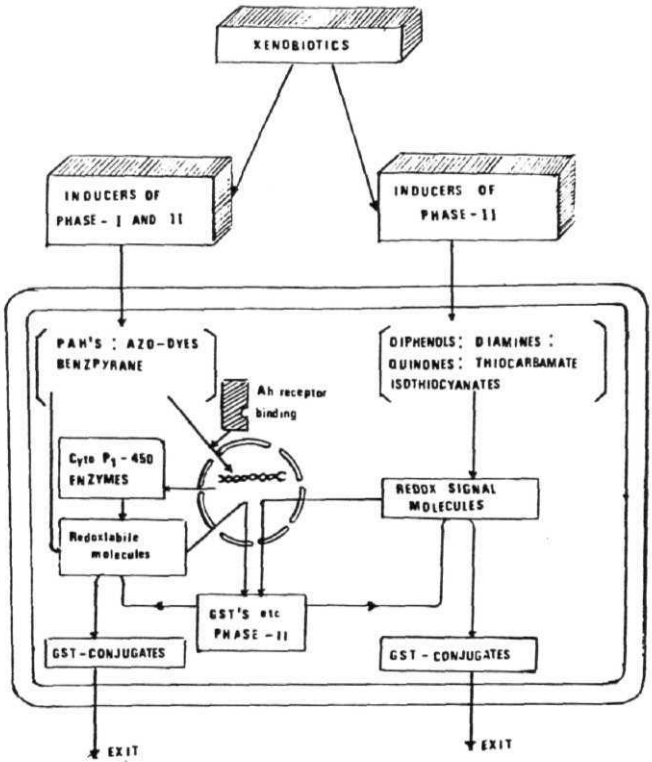


Fig. I: PHASE I AND PHASE II DRUG DETOXIFICATION MECHANISMS

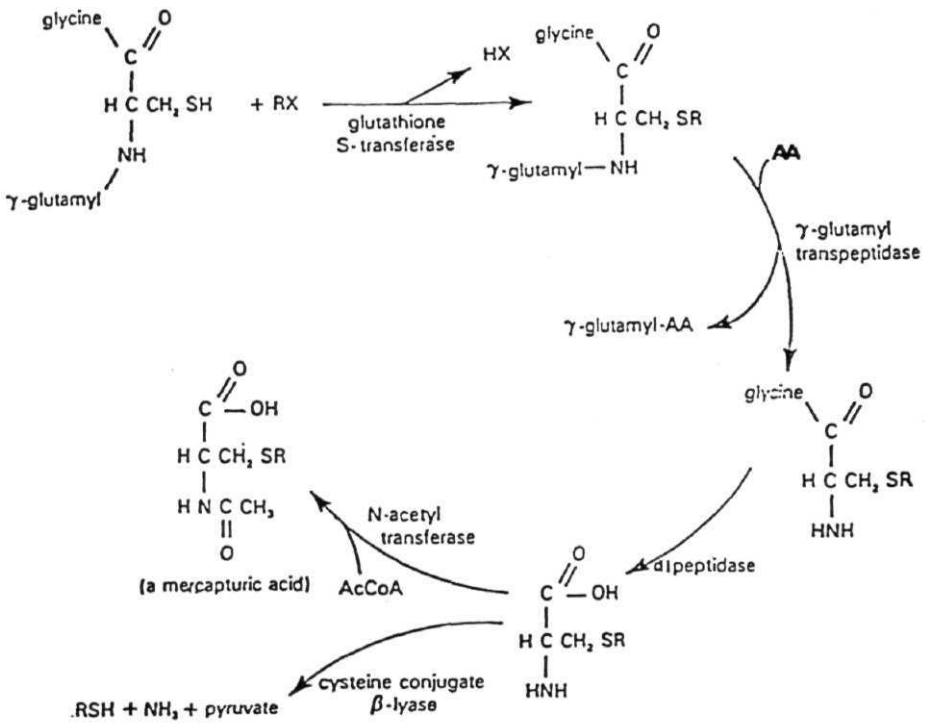


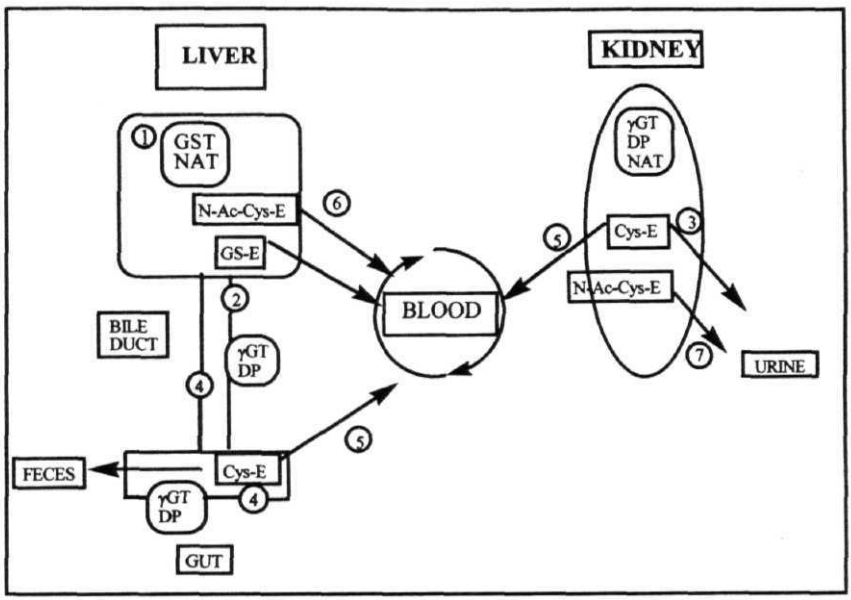
Fig. 2: THE MERCAPTURIC ACID PATHWAY

et al., 1985; Ketterer and Christodoulides, 1994). The cytosolic GSTs from the rat, mouse and man share common structural and catalytic properties. lsozymes from these species were grouped with respect to N-terminal amino acid sequence, substrate specificities, sensitivities to inhibitors and immunological cross reactivity. An additional classification of GST nomenclature is by their subunit resolution on SDS-PAGE (Bass *et al.*, 1977). In this study, both systems of nomenclature will be followed (summarized in Table 1). The genetic relationship among subunits is believed to be as follows: a family- Ya_1, Ya_2, Yc, Yk and Yfetus; μ family- Yb_1, Yb_2, Yn_1, Yn_2 and Yo; π family- Yp; and θ family- subunit 5 and subunit 12. Evolutionarily, cytosolic GSTs were derived from common origin as illustrated by their considerable homology of > 70% according to amino acid sequencing within gene families and 20-30 % homogeneity between gene families. The GST isozymes have generally been found to occur as homodimers and heterodimers of subunits ranging in size from 17 to 28 kDa (Mannervik and Dannielson, 1988). Rat liver contains at least 18 dimers made up of 13 different subunits (Tsuchida and Sato, 1990).

1.1.3.0. Mechanistic aspects of GSTs

The active site of GST is composed of two domains, smaller one for binding of glutathione (G-site) and the larger hydro phobic domain for electrophilic substrate (H-site). All GSH binding sites have a high specificity towards GSH, except for theta class GSTs (Meyer, 1993). Similarly, the affinity of the electrophilic domain is also high for many electrophiles especially 1-chloro-2,4-dinitrobenzene (CDNB), a classical substrate used for most studies involving GSTs.

The exact mechanism of GST-catalyzed reaction is still a subject of debate. The most likely mechanism involves the lowering of pKa of GSH from pH 9.0 to about 6.5 at the active site to form a potent nucleophilic species, the thiokte anion GS". It was hypothesized that a tyrosine residue found at the N- terminus of most cytosolic GSTs is responsible for ionizing GSH (Ji *et al.*, 1992b). Once the GS is



(Curtesy of Hinchman and Ballatori, 1994)

Fig 3 : Current model for the interorgan cycle of mercapturic acid formation; (1) GSH conjugation reactions occur predominantly in the liver, catalyzed by glutathione S-transferases (GST), (2) glutathione conjugates (GS-E) are released from liver into bile or the circulation, (3) renal γ-glutamyltransferase (gGT) and dipeptidase (DP) activities catabolize circulating glutathione conjugates to the corresponding cysteine conjugates (Cys-E), (4) glutathione conjugates released into bile are degraded by gGT activity in the biliary tree and small intestine, (5) cysteine conjugates formed in kidney or gut are released into the circulation and transported back to the liver for acetylation by N-acetyltransferases (NAT), (6) mercapturates (N-Ac-Cys-E) are released from liver and transported to the kidney, and (7) mercapturates cleared by the kidney or those formed by renal N-Acetyltransferase activity are excreted into urine.

Table 1: Rat GST nomenclature based on subunit

Gene family

a		***n***	**G**
1-1 (Ya)	3-3 (Yb,)	7-7 (Yp)	5-5
2-2 **(Yc)**	**4-4 (Yb_2)**		12-12 (Yrs)
8-8 (Yk)	6-6 (Yn,)		
10-10 **(Yc_2)**	9-9 **(Yn_2)**		
	11-11 (Yo)		

From Ketterer and Christodoulides, **1994**.

formed in the active site of GST, it becomes capable of reacting spontaneously by nucleophilic attack with electrophilic xenobiotics that are situated in close proximity. Thus, catalysis by GST occurs through the combined ability of the enzyme to promote the formation of GS" and to bind hydrophobic electrophilic compounds at a closely adjacent site. Several lines of evidence suggest that binding of GSH is accompanied by a conformational change of the protein (Mannervik and Dannielson, 1988). The role of glutathione has been dissected into two components i.e., making the enzyme catalytically competent by inducing a conformational change as well as providing the functional thiol group for the reaction to be catalyzed. Enzymatically, the GSTs function by a sequential mechanism requiring both substrates to be bound before any product is released (Jakobson *et al.,* 1979). Mechanisms concerning the glutathione peroxidase (GSH Px) activity of these enzymes has been hypothesized in a two- step reaction. First, GSH reacts with the peroxide yielding the corresponding alcohol and the sulfenic acid of GSH (GSOH) (Eq. 1).

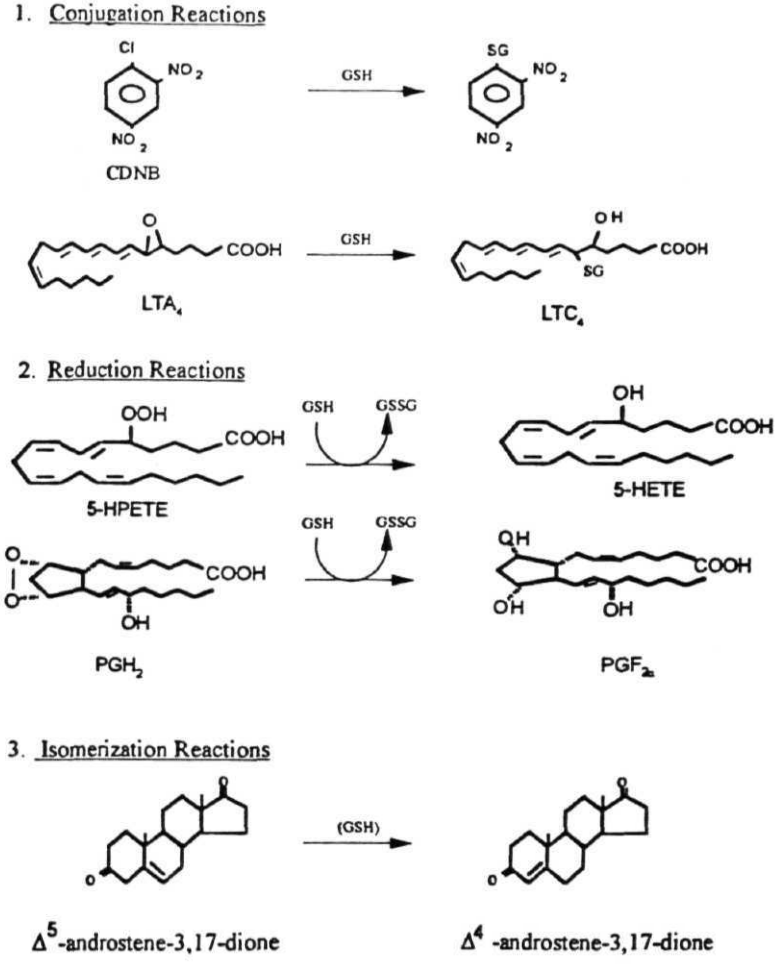
$$ROOH + GSH \rightarrow ROH + GSOH \qquad \text{(Eq. 1)}$$

$$GSOH + GSH \rightarrow GSSG + H_2O \qquad \text{(Eq. 2)}$$

In the second step (Eq. 2), the sulfenic acid reacts with another GSH molecule producing oxidized glutathione (GSSG) and H_2O, which is non-enzymatic. Jakoby and Habig (1980) suggested a simple "proximity effect" in which the GSTs bind GSH and an electrophile and that the electrophilicity of the second substrate then determines the reaction rate.

1.1.4.0. Reactions catalyzed by GSTs

GSTs catalyze 3 major reactions viz. conjugation, oxidation-reduction and isomerization (Fig. 4). GSTs also catalyze the addition reactions towards epoxides including leukotriene A_4 (LTA_4) and arene epoxides (Chasseaud, 1979; Samuelsson, 1983; Chang *et al.*, 1987b). In each reaction GSH functions as the nucleophilic reactant towards an electrophilic substrate. The most sensitive and



4. Binding

In addition to its catalytic activities, GSTs can also non-enzymatically bind numerous compounds such as bilirubin, heme and thyroid hormones.

Fig. 4: The Catalytic and Non-catalytic Functions of GSTs

widely used substrate to study GST is CDNB, a reaction easily analyzed spectrophotometrically. The newly discovered theta class GST, Yrs-Yrs do not react with CDNB but efficiently conjugates reactive sulfate esters formed from carcinogens (Hiratsuka *et al.,* 1990). Majority of substrates for GSTs are xenobiotics and include many known carcinogens in addition to a number of therapeutic compounds.

A second class of reactions catalyzed by GSTs are oxidation-reduction reactions, where GSTs exhibit peroxidase activity, commonly denoted as non-selenium-glutathione peroxidase (non-Se-GSH Px) activity. The substrates that GST reduce include fatty acid, phospholipid and DNA hydroperoxides. As these compounds are generated by lipid peroxidation and oxidative damage to DNA, it has been proposed that GST as well as other GSH-dependent enzymes help combat oxidative stress (Mannervik, 1986). Both a-and 8- class GSTs are particularly high in this GSH Px activity. Detoxification of lipid hydroperoxides by microsomal GSTs can occur *in situ,* whereas detoxification of lipid hydroperoxides by cytosolic GSTs require prior release of fatty acid hydroperoxides by phospholipase A_2 (PLA2) (Tan *et al.,* 1984). A second peroxidation reaction catalyzed by GSTs includes the reduction of the endoperoxide prostaglandin H_2 (PGH_2) to $PGF_{2\alpha}$ (Burgess *et al.,* 1987; Hong *et al.,* 1989; Chang *et al.,* 1990).

GSTs also catalyze reactions of positional isomerization. An example of a GST-mediated reaction is the isomerization of PGH_2 to the biologically active PGE2 and PGD2, the major steps in eicosanoid biosynthesis (Christ-Hazelhof *et al.,* 1976; Burgess *et al.,* 1987), GSTs also catalyze the conversion of A^5-ketosteroids to Δ^4-ketosteroids (Benson *et al.,* 1977).

In addition to their catalytic role, GSTs are known to bind to a number of hydrophobic compounds including heme, bilirubin, and steroids (Vander Jagt *et al.,* 1985). As a result of this binding function, the most abundant GST isozyme from rat liver was originally termed as ligandin, which was later identified as an a-class GST (Habig *et al.,* 1974). The abundance of GST in both rat and human liver

suggests that the binding function may be of even greater significance than its catalytic role (Tipping and Ketterer, 1981).

1.1.5.0. Cytosolic GSTs

In 1961, Booth and co-workers undertook a systematic evaluation of the nature of GSH-conjugation reactions from rat liver. They demonstrated that 82% of the GST activity towards 1,2-dichloro-4-nitrobenzene (DCNB) was found in the soluble fraction, and only 4% located in the microsomes (Booth *et al.*, 1961). Not surprisingly, these resuhs led to widespread examination of those GSTs located in cytosolic fractions.

Glutathione-agarose and S-hexylglutathione-agarose have been extensively used to purify cytosolic GSTs (alpha, mu, pi and sigma classes). Class mu, pi and sigma GSTs are adsorbed efficiently by both glutathione-agarose and S-hexylglutathione-agarose, whereas the alpha class do not display strong affinity for S-hexylglutathione-agarose but most isozymes of this class are efficiently adsorbed by glutathione-agarose (Hayes, 1986).

The cytosolic GSTs in all vertebrate species exist either as homodimers or heterodimers. The multiple cytosolic GSTs have been identified by analytical methods such as SDS-PAGE, isoelectric focussing, western blotting. In recent times, HPLC has been increasingly employed to identify GST subunits (Johnson *et al.*, 1992). Tissue specific expression is one of the characteristic feature of GSTs and the degree of heterogeneity of GSTs and their isozyme profiles vary from tissue to tissue within species (Awasthi and Singh, 1985; Hayes, 1988; Tsuchida and Sato, 1990; Johnson *et al.*, 1992). In addition, sex related differences in the levels of GST activity and subunit composition also has been suggested in mammalian tissues (Hatayama *et al.*, 1986; Carrillo *et al.*, 1991). Male rats had higher concentrations of u class subunits and lower a class subunits compared to female rats (Carrillo *et al.*, 1991). Rat liver shows Ya, Yb and Yc subunits whereas Ya subunit is lacking in brain, heart and lung (Thyagaraju *et al*,

1986b; Johnson *et ah,* 1992). Both 'a' and 'μ' class GSTs are found in low concentrations while 'π' class is the predominant isozyme in lung (Carmichael *et ah,* 1988; Cossar *et ah,* 1990).

1.1.6.0. Glutathione S-transferases in eicosanoid metabolism

Arachidonic acid (AA) is the most abundant polyenoic fatty acid found in the phospholipids of mammalian tissues. It is a biosynthetic precursor of several classes of biologically active eicosanoids. Once arachidonic acid is released from membrane phospholipids, it is metabolized by one of the two pathways- lipoxygenase (LOX) and cyclooxygenase (COX) and produce a spectrum of biologically active compounds termed as eicosanoids (Fig. 5&6). The COX produces prostaglandins (PGs), thromboxanes (TXs), and prostacyclin (PGI), whereas the LOX pathway leads to the formation of leukotrienes (LTs) and lipoxins(LXs). These eicosanoids are extremely potent biologically active compounds with bewildering variety of actions.

The first report implicating GSTs in the metabolism of eicosanoids appeared in 1975 when Cagen and co-workers reported that GSTs mediated conjugation of GSH to PGA_2 (Cagen *et ah,* 1975). A year later, the group led by Christ-Hazelhof using purified GSTs from sheep lung and liver showed that these enzymes were efficient in catalyzing the reduction and isomerization of the endoperoxide PGH_2 to all known PGs (Christ-Hazelhof *et ah,* 1976). When AA becomes available in response to a specific stimulation in a PG metabolizing cell, the first step in PG formation is the PGH synthase catalyzed incorporation of two molecules of oxygen to form PGH2, via the intermediate PGG2 (Hamberg *et ah,* 1974). PGH_2 is a common precursor for the synthesis of PGIs and TXs as well as classical PGs- PGD_2, PGE_2 and $PGF_{2\alpha}$.

The action of isomerase on the endoperoxide moiety of PGH_2 lead to PGE_2 and PGD2 formation whereas reductases act on PGH_2/PGE_2/ PGD2 to yield $PGF_{2\alpha}$. Previously GSTs have been implicated in the biosynthesis of PGs from

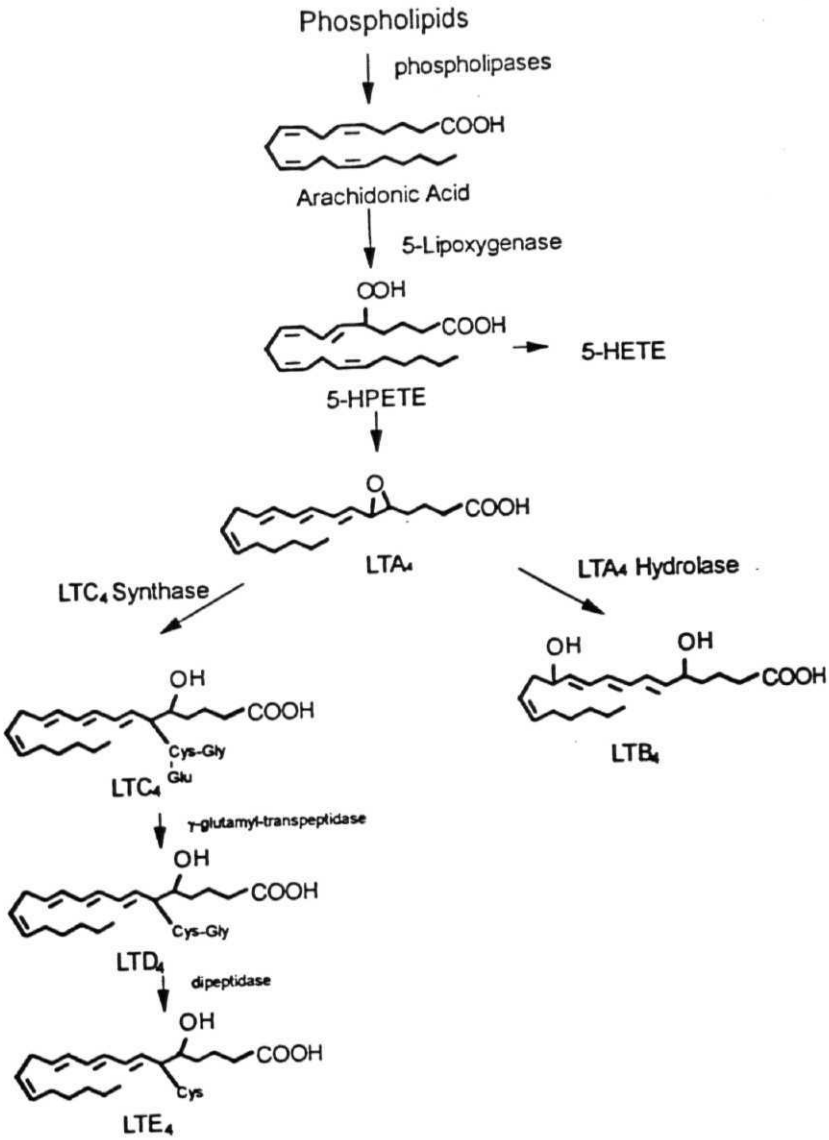


Fig. 5: Leukotriene Biosynthesis

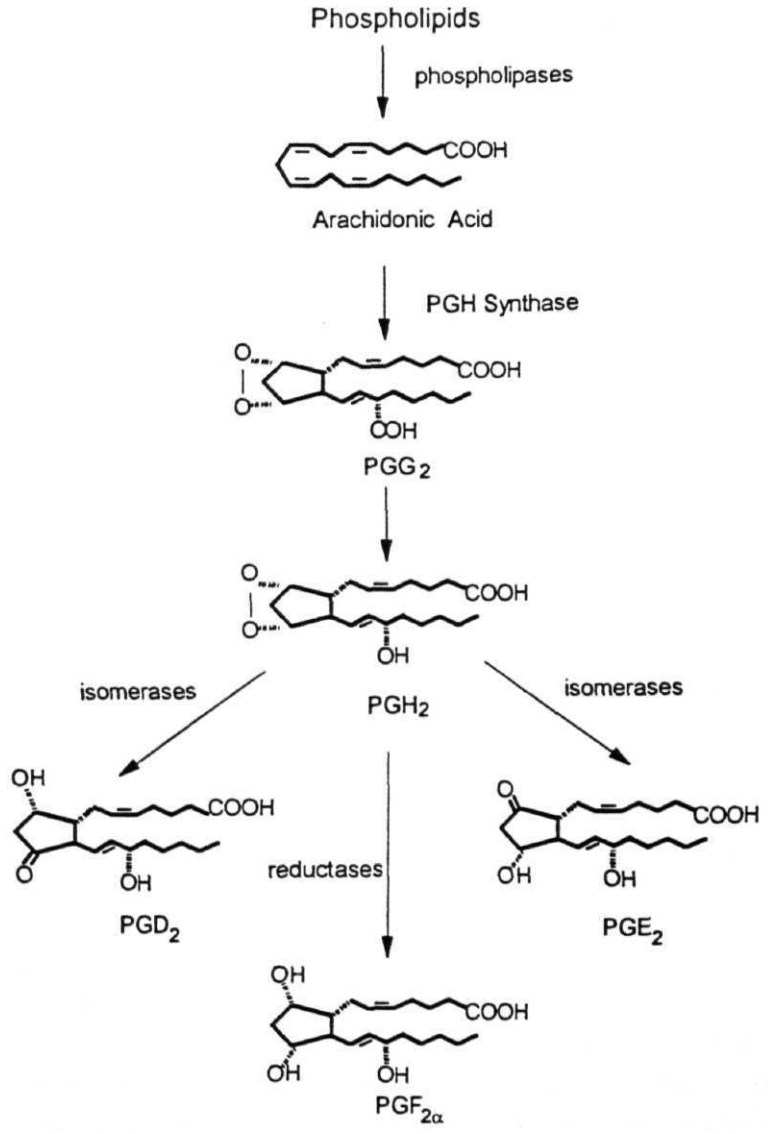


Fig. 6: Prostaglandin Biosynthesis

PGH_2 (Burgess *et al,* 1987; Chang *et al,* 1987a). PGD_2 is formed by the isomerization of the 9,11-endoperoxide to 9-hydroxy and 11-keto groups. This catalytic activity has been demonstrated in cytosol of certain rat tissues-lung, stomach, brain, and skin (Yamamoto, 1982). The formation of PGE_2 is also isomerization reaction converting the endoperoxide to a ketone at C-9 and an alcohol at C-11. The formation of PGE_2 by GST was established in human brains by Ogarachi *et al* (1987) and in rat brain by Thyagaraju *et al* (1986a).

Tissue specific enzymes that result in the formation of products characteristic of a given tissue determine the metabolic fete of PGH_2. PGH_2 is exclusively converted to thromboxane A_2 in platelets catalyzed by thromboxane A synthase, whereas , in vascular epithelium, it is transformed mostly into PGI_2 by PGI synthase. Such classical PGs such as $PGF_{2\alpha}$, PGE_2 and PGD_2 are formed in most tissues but to differing degrees. Thus tissue specificity appears to be an important factor in the regulation of physiological functions. $PGF_{2\alpha}$ is widely distributed in various organs of mammals and shows a variety of biological activities including uterine smooth muscle contractions and luteolysis. GSTs have been implicated in the prostaglandin biosynthesis by virtue of its endoperoxidase activity (Chang *et al.,* 1987a).

In addition to their role in PG biosynthesis, GSTs also have been implicated in LT biosynthesis. The LTs are very reactive compounds involved in hypersensitivity and inflammatory disease processes such as asthma and allergic reactions. Controlling the production of these bioactive lipids is the continuing focus of pharmaceutical research. The major LTs are LTA_4, LTB_4, LTC_4, LTD4 and LTE4. The latter three compounds are collectively referred to as "Slow reacting substances of anaphylaxis" (SRS-A). They are potent bronchoconstrictors in several species including humans, with specific effects on the peripheral airways (Dahlen *et al.,* 1980; Smedgard *et al.,* 1982). The first step in the formation of LTs is the oxidation of unesterified arachidonic acid by 5-lipoxygenase. The product of this reaction, 5-hydroperoxyeicosatetraenoic acid (5-HPETE) may be reduced by various peroxidases producing 5-hydroxyeicosatetraenoic acid

(5-HETE) or alternatively undergo enzymatic/nonenzymatic conversion to the unstable allelic epoxide LTA4. LTA_4 serves as a precursor for the biosynthesis of LTB_4 as well as cysteine containing LTs. The conversion of LTA_4 to LTC_4 by GSH conjugation has been proposed. It is well established that endogenous compounds as well as xenobiotics may form epoxides and that GSH conjugation is a significant route in their biotransformation (Reddy *et al.*, 1982). Thus LTA4 can act as a substrate for GSTs. In several cell lines, the LTA4 to LTC4 reaction has been shown to be catalyzed by GSTs (Hammarstrom and Samuelsson, 1980). LTC_4 is enzymatically transformed into a more potent biological mediator, LTD_4 and LTE4 by sequential action of γ-glutamyl transpeptidase (Tate *et al.,* 1976) and a dipeptidase (Lee *et al.,* 1983). Formation of LTF4 from LTC_4 by the removal of glycine catalyzed by carboxy peptidase A was also demonstrated (Reddanna *et al.,* 1988). Although the exact role of cytosolic GSTs in LTC_4 biosynthesis is unclear, there is evidence for a unique LTC_4 synthase that is distinct from any previously isolated GST (Soderstrom *et al.,* 1988; Penrose *et al.,* 1992).

1.1.7.0. Induction of GSTs as part of adaptive response to oxidant stress

It is apparent that the species, strain, age, sex and organ influence the responsiveness of rodent GST to inducing agents (Hayes and Pulford, 1995). Tissues expressing high activities of GSTs are protected from cytotoxic damage elicited by electrophiles for which the conjugation to GSH is readily catalyzed. Not all GST subunits are induced to the same extent by drugs. Meyer *et al* (1993) examined GST subunit induction by 1,2-dithiole-3-thione in intestine, kidney, liver, lung and stomach and showed that the pattern of induction varies significantly in different organs. GSTs are over-expressed in certain tumor types and measurement of GSTs in serum of pathological specimens can be used to follow the course of the disease and the success of intervention (Primiano and Novak, 1992). The earlier stages of hepatocarcinogenesis are characterized by dramatic increase in the expression of the rat GSTP1 gene. Though this class of GST is ubiquitously distributed, but is found only in relatively small amounts in normal rat liver (Sato, 1989). Measurement of plasma hGSTP1-1 levels has shown that this

protein is dramatically increased in lung cancer (Beckett and Hayes, 1993). Epidemiological studies suggest that individuals who are homozygous nulled at the GSTMl locus may have an increased risk of developing various types of neoplastic diseases, including cancer of lung, bladder, colon and stomach (Seidegard and Pero, 1985). hGSTM1a-1a and hGST M1b-1b are active towards certain epoxides of poly aromatic hydrocarbons (PAH) found in cigarette smoke and other combustion products which makes efforts focused on the area of lung cancer. hGST M1-1 deficiency also contributes to individual susceptibility to asbestos-induced pulmonary disease (Smith *et al.,* 1994). Both phenobarbital and 1,2-dithiole-3-thione are potent GST inducers in male than in female sprague dawley rats (Meyer *et al.,* 1993), whereas 3-methylcholanthrene is a slightly better inducer of hepatic GST in female than male rats (Igarashi *et al.,* 1987). GSTs also participate in the development of drug resistance in patients undergoing chemotherapy (Hayes and Pulford, 1995).

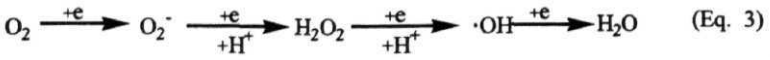
GST induction as a part of an adaptive response mechanism to xenobiotic stress is widely distributed in nature (Vos and Van Bladderden, 1990). It in turn involves the induction of many drug- metabolizing enzymes (Talalay and Spencers, 1990). Collectively, these detoxification enzymes provide protection against a diverse spectrum of harmful compounds. Besides providing protection against chemicals of foreign origin, GSTs are involved in protection against oxidative stress. Hence an attempt is made to study the antioxidant defense mechanism of GSTs in liver and lung tissues of rats subjected to oxidative stress. Oxidative stress can be defined as the inability of an organism to protect tissues from free radicals generated by exogenous and endogenous toxic substances. Oxidative stress can be induced by either hyperoxic conditions, H_2O_2, exercise, chemical inducers or vitamin E (vit.E) and selenium (Se) deficiency. GSTs of μ family are known to be induced by the endogenous toxicants of oxidative stress such as free radicals generated by xanthine-xanthine oxidase reaction (Murata *et al.,* 1990). Rushmore *et al* (1991) showed the induction of GST Ya mRNA by hydrogen peroxide. Oxidant stress, in the form of swimming exercise induced Ya_2 subunit in

the hepatic tissue of female rats (Veera Reddy *et al.,* 1995). Selenium deficient rats are generally exposed to increased intracellular levels of H_2O_2 because of lack of Se-GSH Px. This resulted in over-expression of hepatic GST isozymes (Arthur *et al.,* 1987) as a compensatory mechanism. The a family of GST-Ya, with highest peroxidase activity (Tu and Reddy, 1985), is known to be induced during Se deficiency (Chang *et al.*, 1990).

1.2.0.0. Oxygen toxicity

Oxygen (O2) is indispensable to life, except for certain anaerobic microorganisms. The principal function of O_2 in biological system is to serve as the terminal oxidant in cellular respiration, thus providing energy for the organism. However, O_2 as a double edged sword, induces toxic effects above atmospheric conditions (21%). Exposure of adult humans to pure O_2 for as little as 6 hours can cause chest soreness, cough and sore throats in few people (Halliwell, 1994) and further exposure leads to irreparable damage of the lung alveoli. The lung tissue is one of the organs exposed to the highest tissue pO_2 under normal conditions and is also the first target of oxygen toxicity *in vivo* at normobaric oxygen partial pressure (Johnson *et al.,* 1981; Jamieson *et al.,* 1986). Severe morphological and functional alterations in the lung are caused by inhaling elevated concentration of oxygen often resulting in death (Crapo *et al,* 1980a). The damaging effects of oxygen on aerobic organisms vary considerably with the type of organism used, its physiological state, diet and age. Oxygen toxicity is also influenced by the presence in the diet of varying amounts of vitamins, heavy metals, antioxidants and poyunsaturated fatty acids (PUFAs). The earliest explanation for the toxicity of O_2 was related to its effects on key enzymes associated with energy metabolism. Although O_2 inhibits certain enzymatic activities, this proved insufficient to explain the mechanism of O_2 toxicity. Gerschman and co-workers (1954) and Harman (1956) hypothesized that the possible cause of O_2's dark side may not be O_2 itself, but rather due to the reactive products produced during partial reduction of O_2. It has long been known that metabolism of O_2 to water occurs by sequential addition

of four electrons (Eq. 3). However, in the event of incomplete tetravalent reduction, O_2 is converted to superoxide anion radical (O2), hydrogen peroxide (H_2O_2), and/or the highly reactive hydroxyl radical (OH), all of which are known as reactive oxygen species (ROS) (Scandalios, 1993).



(Eq. 3)

In addition to being ROS, both (V and OH are also free radicals. Free radicals are species capable of independent existence and contain one or more unpaired electrons. It is the presence of these unpaired electrons that confer the high reactivity often associated with free radicals. ROS not only includes oxygen radicals but also contain non-radicals such as H_2O_2, singlet oxygen and hypochlorous acid (HOCl).

1.2.1.0. Sources of Free radicals

Exposure of tissue to free radicals leads to tissue damage with lipids, proteins, and DNA as the targets for free radical injury. Although the ROS have been involved in various tissue pathologies, they are an integral part of normal aerobic metabolism. Phagocytic cells produce ROS through the NADPH-Oxidase system to destroy invading microorganisms (Forman and Thomas, 1986). The damage produced by superoxide radicals liberated from phagocytic cells can spill beyond the integrated target and produce injury to surrounding tissues. Peripheral cells such as fibroblasts and lymphocytes use O_2^- as a growth regulator (Burdon, 1992). Numerous catalytic cytosolic enzymes also contribute to generation of O2" and OH and partially to H_2O_2. Xanthine oxidases are well-known sources of O_2^- generation as indicated by *its* ability to reduce cytochrome C (McCord and Fridovich, 1968). For example cytochrome P-450 and cyclooxygenase all generate ROS through their normal catalytic cycles. Recently, yet another free radical, nitric acid (NO), has been implicated as a regulator of physiological processes (Moncada *et a!.*, 1991). ROS are formed endogenously and exogenously (Halliwell and Gutteridge, 1989). Autooxidation reactions also

generate $O_2^{\cdot}$. Organic compounds such as ascorbate, flavins and tetrahydrofolate, which readily undergo one electron oxidation produce $O_2^{\cdot}$ (Fridovich 1989 and references therein). Endogenous sources of H_2O_2 include reactions involving various oxidases such as xanthine and amino acid oxidases as well as dismutation of $O_2^{\cdot}$. Regardless of the precise role free radicals or ROS play in tissue damage, most studies suggest that biological macromolecules are the primary targets for their action. Phospholipids, the major constituents of biological membranes are highly susceptible to oxidative attack (Halliwell and Chirico, 1993).

1.2.3.0. Lipid Peroxidation

Lipid peroxidation is a chain-reactive process with three distinct stages. These stages are initiation, propagation and termination (Table 2). It is initiated by abstraction of a H atom by those species with sufficient oxidation potential towards divinyl methane groups of PUFAs. These oxidizing species include such free radicals as OH, $ROO^{\cdot}$, RO and NO or ROS such as singlet oxygen ($^1O_2$) and O_3. Neither O_2, $O_2^{\cdot}$ nor H_2O_2 are themselves sufficiently reactive to abstract $H^{\cdot}$ atoms from PUFAs, and therefore cannot initiate lipid peroxidation (Halliwell and Gutteridge, 1989). These ROS can, however, initiate lipid peroxidation, in the presence of redox-active transition metals. For example, H_2O_2 undergoes homolytic cleavage in the presence of transition metals producing perhaps the most reactive oxidizing free radical, OH (Eq. 4). This reaction is often referred to as the Fenton reaction. The OH is also formed by the transition metal assisted Haber-Weiss reaction (Eq. 5) (KoppenoL 1990).

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH + OH \quad \text{(Eq. 4)}$$

$$O_2^{\cdot} + H_2O_2 \rightarrow O_2 + OH + OH \quad \text{(Eq. 5)}$$

Transition metals in particular iron and copper, stimulate membrane oxidative damage at the initiation and/or propagation steps. Another initiator of lipid peroxidation is $^1O_2$, which causes damage to both plant and animal tissue (Cadenas, 1984).

Table 2: The 3 stages of lipid peroxidation

RH $\rightarrow$ R$^{\cdot}$	Initiation	(Eq. 6)
R$^{\cdot}$ + O_2 $\rightarrow$ ROO	Propagation	(Eq. 7)
ROO$^{\cdot}$ + R'H $\rightarrow$ ROOH + R$^{\cdot}$		(Eq. 8)
ROO$^{\cdot}$ + ROO$^{\cdot}$ $\rightarrow$ ROOR + O_2	Termination	(Eq. 9)
ROO$^{\cdot}$ + vitamin E $\rightarrow$ ROOH + vitamin E$^{\cdot}$		(Eq. 10)

The free radical attack on the membrane PUFA leads to the abstraction of a hydrogen atom resulting in a carbon-centered lipid radical (Eq. 6). In the aerobic environment of the membrane bilayer, the lipid radical reacts with the molecular oxygen at a diffusion controlled rate (10^9 M^{-1} s^{-1}), yielding a lipid peroxyl radical with rearrangement of double bonds to yield a conjugated diene (Eq. 7). Peroxyl radicals are, in turn, capable of abstracting a second hydrogen atom from an adjacent PUFA, thereby leading to propagation of the chain reaction (Eq. 8). Alternatively, a chain breaking antioxidant such as vit.E scavenges the peroxyl radical and prevents further membrane oxidative damage (Eq. 10). Regardless of the target molecule, the reaction with the lipid peroxyl radical usually results in a formation of a lipid hydroperoxide. With the propagative nature of lipid peroxidation, a simple initiation event produces many lipid hydroperoxides. The membrane PUFA content and as well as O_2 availability both control the extent of propagatioa

In the presence of transition metals, lipid hydroperoxides undergo decomposition and produce more reactive alkoxyl or peroxyl radicals. A second fate of alkoxyl and peroxyl radicals is the cleavage of carbon-carbon bonds, resulting in the formation of secondary decomposition products including alkenals, hydroxyalkenals, and alkanes (Esterbauer *et al.*, 1990). Termination of lipid

peroxidation can occur by coupling of peroxyl radicals producing non-radical products and O_2 (Eq. 9) or free radical scavenging by antioxidants (Eq. 10).

Secondary by-products of lipid peroxidation may be responsible for much of the free radical-mediated damage occurring during oxidative stress. ROS are involved in the mediation of a variety of pathophysiological conditions. Biological systems, however, are equipped with well developed antioxidant defense mechanisms to either regulate the production of ROS or to convert the highly reactive oxygen species into less reactive Lipid peroxidation may also be responsible for liver necrosis in rats, white muscle disease in sheep and encephalomalacia in chicks. These abnormalities occur as a result of a dietary deficiency of two important antioxidants, vit.E and Se. Lipid peroxidation occurs in tissues of rats fed on vit.E deficient diet and is increased by concomitant Se deficiency (Tiidus *et ah,* 1993; Awad *et ah,* 1994).

1.3.0.0. Antioxidant Defense Systems

Under conditions of normal metabolic conditions, tissues have adequate defense mechanisms to minimize damage caused by oxidative stress. Antioxidants, can be defined as "compounds that protect biological systems against the potentially harmful effects of processes or reactions that can cause excessive oxidations" (Krinsky, 1992). Mammalian cells are usually equipped with both enzymatic and non-enzymatic antioxidants. The enzymatic antioxidant defenses include superoxide dismutase (SOD), catalase, Se-GSH Px, GSTs and glutathione reductase. The non-enzymatic biological antioxidants are vit.E, ascorbic acid, carotenoids, GSH, cysteine and uric acid. Various antioxidants being both lipid and water soluble, are found in all parts of cells and tissues, although each specific antioxidant often shows a characteristic distribution pattern. Deficiency of nutritionally derived antioxidants such as vitamin C and E and excess production of ROS favours oxidative stress. The characterization of biological antioxidant defense constitutes one of the most active fields of free radical research today (Halliwell, 1990).

1.3.1.0. Non-enzymatic defense systems

1.3.1.1. Glutathione

The y-glutamyl cycle serves a diverse series of critical cellular defensive functions including free radical scavenging, detoxification of electrophiles and the maintenance of thiol-disulfide status (Fig. 7). GSH is the key compound of the y-glutamyl cycle (Sen *et al.,* 1992). It is the most abundant nonprotein thiol in eukaryotic cells with several important biological functions. GSH has been known as a substrate for GSTs and GSH Px, enzymes involved in the detoxification of xenobiotic compounds and reduction of hydroperoxides (Meister, 1991; Hwang *et al.,* 1992). GSH also acts as an efficient scavenger of superoxide radical ($O_2^{\cdot}$), hydroxyl radicals (OH) and singlet oxygen (Halliwell and Gutteridge, 1989). It is actively involved in the recycling of α-tocopheryl and semihydroascorbate radicals (Halliwell and Gutteridge, 1989), protein conformation, cytoskeletal organization (Larsson *et al.,* 1983), modulation of enzyme activity (Gilbert, 1984) and acts as a reservoir for cysteine (Tateishi *et al.,* 1977). Reduced GSH is present at a relatively high concentrations in the body, ranging from ~ 1 mM in skeletal muscle to 10 mM in eye lens. The intracellular ratio of GSH to GSSG (GSH/GSSG) is usually maintained at a relatively high level (> 10) by a glutathione reductase catalyzed recycle pathway. Liver plays a central role in the synthesis as well as complex inter-organ homeostasis of GSH (Beatty and Reed, 1980; Deneke and Fanburg, 1989). In the cell GSH is synthesized by two cytoplasmic enzymes namely γ-glutamylcysteine synthetase (GCS) and GSH synthetase. GCS is the rate limiting enzyme in y-glutamyl cycle (Richman and Meister, 1975).

The interorgan homeostasis of GSH is summarized in figure 8. In addition to export of hepatic GSH into the plasma and uptake by extrahepatic tissues, hepatic GSH is also released into the bile (Lauterburg *et al.,* 1984). GSH is exported from tissues such as lung and liver in its oxidized form, glutathione disulfide (GSSG), and as glutathione conjugates (GSX). GSSG is produced when peroxides are detoxified by GSH Px (Fig. 7, step 9) and is recycled back to the reduced form by glutathione reductase (Fig. 7, step 12) at the expense of NADPH.

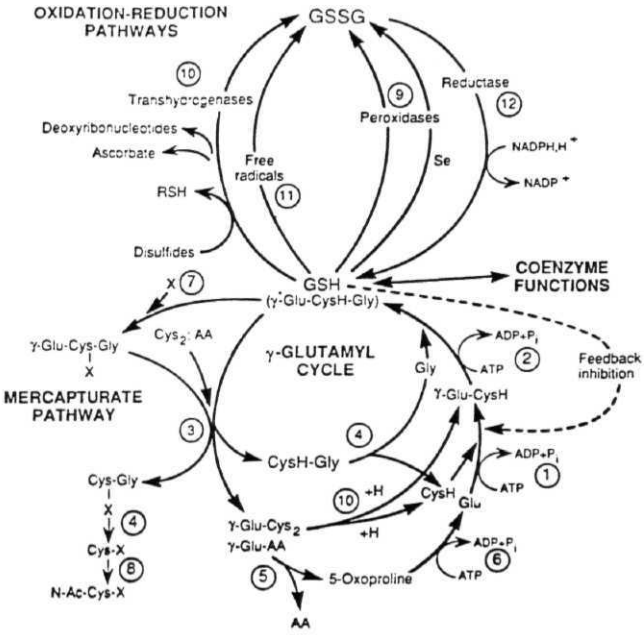


Fig. 7: The Metabolism of Glutathione

(Courtesy of Meister and Anderson, 1983)

However, in the absence of adequate reducing equivalents, cellular thiol balance is preserved by export of GSSG and uptake by the kidney (McIntyre and Curthoys, 1980). GSX is formed when reactive metabolites conjugate with GSH spontaneously or enzymatically by the GSTs (Fig. 7, step 7). Subsequent removal of the γ-glutamyl moiety by y-glutamyl transpeptidase (GGT) (Fig. 7, step 3) and cleavage of glycine by a dipeptidase yields a cysteinyl conjugate (Fig. 7, step 4). In the kidney, the cysteinyl conjugate is N-acetylated and excreted as mercapturic acid (Fig. 7, step 8) (DeLeve and Kaplowitz, 1990). γ-Glutamyl amino acids formed in this way are converted to 5-oxoproline and the corresponding free amino acids by y-glutamyl cyclotransferase (Fig. 7, step 5). 5-Oxoproline is converted to glutamate in the ATP-dependent reaction catalyzed by 5-oxoprolinase (Fig. 7, step 6).

GSH serves as an antioxidant by reacting directly with free radicals (Fig. 7, step 11) and by providing substrates for GSH Pxs (Fig. 7, step 9) and GSH transhydrogenases (Fig. 7, step 10). The enzymes of the GSH cycle and their locational specificity provide the framework for intracellular maintenance of tissue GSH concentrations and interorgan transport of GSH. Toxic oxygen intermediates arising from both normal cellular metabolism and exogenous oxidants are removed by GSH Pxs and other GSH-related detoxification mechanisms (Coles and Ketterer, 1990; Jain *et al.*, 1992). GSH thus plays a primary role in protection of tissues against oxidative injury (Deneke and Fanburg, 1989). A number of experimental observations show an association between decreased cellular GSH and increased susceptibity of lung cells to damage by oxidants (Deneke and Fanburg, 1989; Martensson *et al.*, 1989). In contrast, conditions that enhance cellular GSH supply are associated with resistance to oxidant damage (Brown *et al.*, 1992). GSH status can be affected by oxidative stress by the metabolism which include exposure of the cell to high concentrations of hydroperoxides or electrophilic compounds or by inhibition of the enzymes of GSH biosynthesis (Reed, 1990). Any one of these scenarios can result in the depletion of intracellular GSH concentrations.

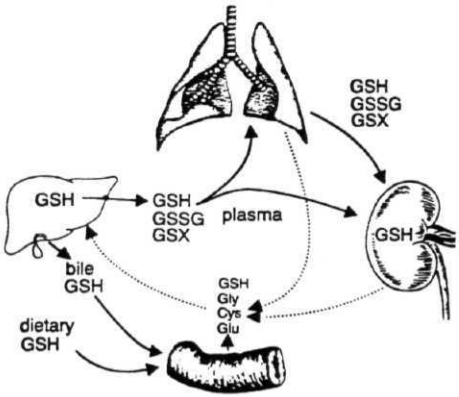


Fig. 8: Interorgan homeostasis of GSH
(adapted from Deneke and Fanburg, 1989)

1.3.1.2. Vitamin E

The primary lipophilic antioxidant located in membranes and lipoproteins is vit.E (Taylor *et al.,* 1976). A major biological function of α-tocopherol is to act as a lipid antioxidant and free radical scavenger that is capable of terminating chain reactions among PUFA molecules. The free radical scavenger action of α-tocopherol depends on the ability of donation of the phenolic hydrogen atom to a fatty acyl free radical, which resolves the unpaired electron of the radical so that free radical attack on further molecules of unsaturated fatty acid is prevented. The primary oxidative product of vit.E in this reaction is tocopheroxyl radical and eventually to a-tocopherol quinone (McCay and King, 1980; Diplock 1985). The quinone can be reduced back to alcohol either by ascorbic acid (Niki *et al.,* 1982) or possibly enzymatically using the reducing equivalents from GSH and NADPH (Tappel, 1962) (Fig. 9). The efficiency of vit.E in protecting against lipid peroxide damage is remarkable considering its concentration in cell membranes. Besides its role in nonenzymatic lipid peroxidation, vit.E was shown to be a potent inhibitor of lipoxygenase (Reddanna *et al.,* 1985; 1989) and aids in the reduction of hydroperoxides (Cucurou *et al.,* 1991). Lung eicosanoid synthesis was shown to be affected by vit.E (Meydani *et al.,* 1992). Vitamin E inhibits some of the reactions leading to the formation of lipoxygenase and cyclooxygenase products (Panganamala and Cornwell, 1980) and also inhibits platelet aggregation *in vitro* and *in vivo* (Stuart, 1982). Vitamin E deficiency, on the other hand depressed synthesis of PGF in testis (Carpenter, 1981) and muscle (Chan *et al.,* 1980). Vitamin E is known to significantly protect lung tissue from damages after NO_2 or O_3 exposures (Elasayed *et al.,* 1988) and tobacco smoke (Arriess *et al.,* 1988). Alterations of vit.E status has been associated with the development of cardiovascular disease (Gey *et al.,* 1987) and impaired immune response (Panush and Delafuente 1985). Animal research has demonstrated that a high PUFA intake increases the vit.E requirement (Horwitt, 1986).

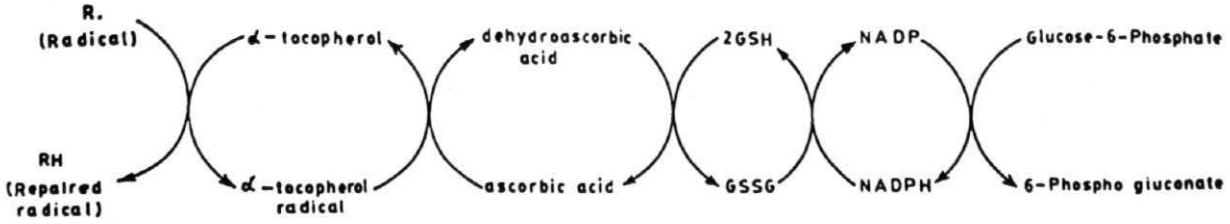


Fig. 9. The recycling of tocopherol, ascorbic acid, glutathione and NADPH

A wide variety of pathological alterations occur in animals and humans with Se and vit.E deficiency. Necrosis of smooth muscle of gizzard and intestine may be a prominent lesion in turkey, ducklings and quail (Van Vleet 1982). Ultrastructural study of the early alterations in rat liver in response to Se-vit.E deficiency showed disruption of hepatocyte plasma membranes at the sinusoidal border (Svoboda and Higginson, 1963). Other Se-vh.E deficiency lesions include gastric ulceration, exudative diathesis, encephalomalasia, pancreatic damage and testicular necrosis. The biochemical pathogenesis of Se-vh.E deficiency diseases has become clearer with the understanding of cellular antioxidant defense systems (Freeman and Crapo, 1982; Hammond and Hess, 1985).

1.3.2.0. Enzymatic antioxidant defense systems

1.3.2.1. Superoxide dismutase (SOD)

SOD occurs in different forms, depending on the cellular fraction (Fridovich, 1989). The cytosolic enzyme is a Cu-Zn requiring enzyme, whereas mitochondrial form requires Mn for activity. The SODs may play a role against O_2^- generating systems such as xanthine/xanthine oxidase. The H_2O_2 produced via SOD (Eq. 11) is readily reduced to water by catalase or more importantly by various GSH Pxs present in the cell

$$2H^+ + 2O_2 \rightarrow H_2O_2 + O_2 \qquad \text{(Eq. 11)}$$

1.3.2.2. Catalase

Catalase is a heme-containing protein found predominantly in the peroxisomes (Halliwell and Gutteridge 1989). *In vitro,* catalase has been demonstrated to inhibit Fe^{+2}/H_2O_2-induced lipid peroxidation (Bucher *et ed.,* 1983). Catalase and Se-GSH Px reduce H_2O_2 to water (Eq. 12&13), whereas SOD converts O_2^- to H_2O_2 (Eq. 11).

$2H_2O_2 \rightarrow 2\ H_2O + O_2$ (Eq. 12)

$H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG$ (Eq. 13)

1.3.2.3. Glutathione peroxidase

Selenium as an essential component of GSH Px, plays a critical role in protecting aerobic organisms from oxygen radical-initiated cell injury. The nutritional essentiality of selenium was first recognized in 1957 by Schwartz and Foltz. In 1973 Rotruck *et al* and Flohe *et al* discovered that Se is an essential structural component of GSH Px. Subsequently, it was demonstrated that Se, in the form of selenocysteine ([Se]cys) residue, is part of the catalytic site of GSH Px (Landstein *et al.,* 1981). This lead to a broader conception of GSH Px as an integral part of the cellular antioxidant system which protects the cell from oxidative damage (Tappel, 1980). GSH Px is located in the cytosol and mitochondria] matrix of many cells. It is a tetramer of identical subunits, molecular weight ranges from 19 to 23 kDa with each monomer containing a gram atom of Se in the form of ([Se]cys) (Landstein *et al.,* 1979).

Se-GSH Px catalyzes the two electron reduction of H_2O_2 as well as several organic hydroperoxides as shown in equations 13 & 14 respectively. Thus, this enzyme differs from a group of Se-independent GSH Pxs (non-Se-GSH Px), the GSTs, that can use the latter only (organic hydroperoxides) as peroxide substrates.

$2\ GSH + ROOH \rightarrow GSSG + ROH + H_2O$ (Eq. 14)

Although this enzyme exhibits a broad specificity towards the peroxide substrate, it is very specific for GSH as a hydrogen donor. Approximately 36% of the total Se is associated with the Se-GSH Px in rat liver (Tappel *et al.,* 1984). The enzyme activity is significantly decreased in the absence of dietary Se, and the specific activity of Se-GSH Px varies as a logarithmic function of Se in the diet (Chow and Tappel, 1974). Se-GSH Px is also involved in the reduction of fatty acid hydroperoxides and cyclic endoperoxides generated during the production of

prostaglandins, leukotrienes and related compounds via COX and LOX pathways (Maddipati and Marnett, 1987; Brucchausen *et al,* 1988). As a result of its action on the reduction of hydroperoxides, it is known to be involved in the inhibition of COX and LOX enzymes (Reddanna *et al,* 1989). Therefore, the modulation of Se-GSH Px activity by dietary Se is of great interest because it plays an important role both in the cell defense against oxidative damage and in the regulation of PG and LT biosynthesis (Hong *et al,* 1989; Reddanna *et al.,* 1989). Since Se levels are highly correlated with the Se-GSH Px activity, this enzyme is often used as a biochemical marker of Se status in animals. Non-Se-GSH Px activity associated with the GSTs significantly induced during Se deficiency (Lawrence *et al.,* 1978; Chang *et al.,* 1990), which catalyzes the reduction of organic hydroperoxides only. Selenium deficiency results in loss of GSH Px activity (Hill *et al.,* 1987; Ji *et al.,* 1988), increased plasma GSH concentration (Hill *et al,* 1987), and increased GSTs in liver (Hill *et al.,* 1987; Chang *et al.,* 1990), skeletal muscle (Ji *et al.,* 1988) and myocardium (Charles Kumar *et al,* 1992; Ji *et al.,* 1992a).

Todate, four Se-GSH Pxs have been characterized 1. classical GSH Px I (Flohe *et al.,* 1973; Rotruck *et al,* 1973); 2. phospholipid hydroperoxide GSH Px, (PHGSH Px) (Ursini *et al,* 1982); 3. plasma GSH Px, (GSH Px-P) (Maddipati and Marnett, 1987) and 4. gastrointestinal GSH Px, (GSH Px-GI) (Chu *et al.,* 1993). Similar substrate specificites are found for the peroxidases - GSH Px I, GSH Px-P, and GSH Px-GI. All catalyze the reduction of H_2O_2, ter-butyl hydroperoxide, cumene hydroperoxide (CHP) and linoleic acid hydroperoxide (Esworthy *et al,* 1991). Low levels of Se are regularly and significantly correlated with the severity of hepatic lesions observed in liver disease and alcoholism (Korpela *et al,* 1985). Also an absolute or relative deficit of Se has been implicated in the development of cancers (Ostero and Prellwitz 1990). The anticarcinogenic action of Se in animal models is well established (Ip and Hayes, 1989; Ip and Ganther, 1990; Lei *et al.,* 1990). The most important human deficiency disease responsible to Se is Keshan's disease, which shows cardiomyopathy (Whanger, 1989). Se toxicity is well

characterized in animals and humans by neurological, hoof and hair alterations (Crintion and O'connor 1978).

1.3.2.4. Glutathione reductase

The catalytic activity of glutathione reductase has a central role in the biochemistry of GSH. It serves to maintain GSH essentially completely in its reduced form (Meister and Anderson, 1983).

$$GSSG + NADPH + H^{+} \rightarrow 2\ GSH + NAD^{+}$$

The intracellular ratio of GSH, usually maintained by GSH recycle pathway protects the erythrocytes from H_2O_2-induced oxidative stress in new born babies (Clashen *et al.,* 1992). Exhaustive exercise is also known to increase glutathione reductase in rat skeletal muscles (Ji and Fu,1992).

1.4.0.0. Vitamin E, selenium and arachidonic acid cascade

Vitamin E and Se-GSH Px could play the more specific role of modulating the enzymic oxidation of AA as shown in the figure 10. The immediate oxygenation products of the enzymatic and nonenzymatic oxidation of AA include the formation of HPETE and cyclic endoperoxides. These products both serve as precursor for the biosynthesis of PG and LT, and have significant influence on the enzymes associated with the AA cascade. Se-GSH Px reduces the reactive fetty acid hydroperoxide (FAHP) and thus influences both the enzyme activities and product profiles.

The free radicals are an integral part of both the COX catalyzed reaction and LTA_4 biosynthesis. Vitamin E interferes with the essential radicals involved with the catalytic cycles of LOX reaction, thus influencing product formation. In addition, vit.E can protect the LOX from its self catalyzed iuactivation by the free radicals generated during this reaction (Lands *et al.,* 1984). Similarly, vit.E could also modulate the COX catalyzed reaction in which the essential role of free radicals has been established. The COX is a heme requiring enzyme having both

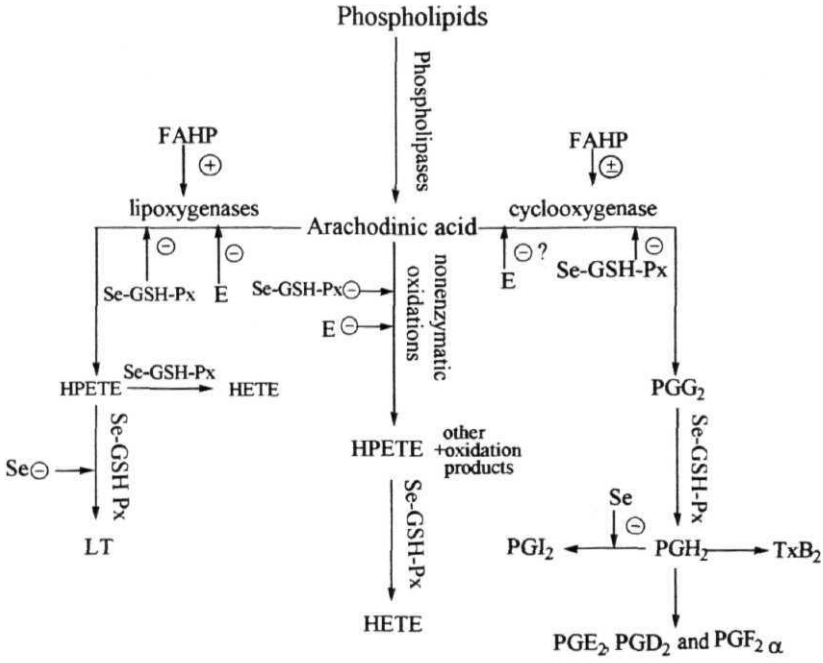


Fig 10 : The proposed role of vitamin E and selenium in the arachidonic acid cascade E , vitamin E, Se, selenium; Se-GSH-Px, selenium-dependent glutathione peroxidase; ⊖, inhibition; ⊕ ,activation; 0 ?, controversial reports, ⊕ , activates at low concentration and inhibits at high concentration

oxygenase and peroxidase activities. The peroxidase reaction of COX generates free radicals which can co-oxidize a host of xenobiotics, including precarcinogens. Selenium, as an integral part of Se-GSH Px is known to involve in this reaction by reducing the endoperoxide to less reactive alcohols (Whelan *et al.,* 1986). Vitamin E as a chain breaking antioxidant has a role in regulating the enzymatic production of these endoperoxides. Thus by removing FAHP activators and essential radicals, Se-GSH Px and vit.E could play a major role in regulating the biosynthesis of PG and LT (Fig. 10) (Panganamala and Cornwell. 1980; Reddy *et al.,* 1988; Reddanna *et al.,* 1989). Vitamin E is a potent inhibitor of LOX (Reddanna *et al.,* 1985; 1989) and the mechanism appears to be not based on its antioxidant but by its peroxidase activity (Cucurou *et al.,* 1991). Non-Se-GSH Px associated with GSTs also reduces FAHPs but ~ 50 times less efficient than Se-GSH Px (Hong *et al.,* 1989). Thus, LT biosynthesis by way of 5-LOX pathway may be partly controlled by cellular FAHP levels, which is in turn under the influence of Se-GSH Px and non-Se-GSH Px.

1.5.0.0. Present scope of the work

Oxygen free radicals generated during normal cellular metabolism are detoxified effectively by efficient antioxidant defense system of the ceD (Freeman and Crapo, 1982). However these defense mechanisms are far from perfect, especially under nutritional deficiency and/or oxidative stress, where the rate of oxygen radical generation may exceed the ability of the system to detoxify free radicals, resulting in tissue damage. Although liver is equipped with abundant antioxidant enzymes and other scavenging systems, the high metabolic rate and central role in detoxification make it one of the targets for free radical damage in the body, moreso under impaired antioxidant defenses. Lung is one of the organ exposed to the highest tissue pO_2 under normal conditions and it is possible that oxygen radicals are of special importance during oxidative stress in the lung tissue. In general, the cellular free radical defense systems play an important role in preventing a radical-mediated tissue damage. Vitamin E as a scavenger of free radicals and potent inhibitor of lipoxygenases and Se as an integral part of

Se-GSH Px, have the potential to protect the tissue from the oxidative stress. GSTs, represent one of the most important detoxifying systems in the liver and lung for numerous endogenous as well as exogenous toxicants. The role of GSTs in the detoxification of a number of xenobiotics has been extensively studied. However there is not much emphasis on the involvement of GSTs in the detoxification of endogenous toxicants especially on those formed during oxidative stress. Hence in the present study an attempt is made to study the antioxidant defense mechanisms in the lung and hepatic tissues of rats subjected to oxidative stress by feeding on diets deficient in vit E and Se. Among the antioxidant defenses particular attention is laid on glutathione metabolism, and glutathione S-transferases. In order to understand the physiological role of GSTs expressed during oxidative stress, the substrate specificities of individual isozymes with special reference to the reduction of organic peroxides and biosynthesis of eicosanoids was undertaken.

Wistar strain female albino rats at weanling stage were fed with vit.E and Se deficient (-E+Se, +E-Se and -E-Se) and supplemented (+E+Se) diets for a period of 13 weeks to impair and enhance antioxidant defenses respectively in the animals. The vit.E and/or Se deficient diets were chosen as they were known to induce oxidative stress in the animals. The first chapter, therefore, includes experiments designed to test the effectiveness of dietary treatment in inducing nutritional deficiency as well as oxidative stress in the rats. This chapter also includes the data on γ-glutamyl cycle enzymes to assess the compensatory defense mechanisms in the glutathione metabolism developed in liver and lung tissues in response to induced oxidative stress. In view of the induction of GSTs observed in vit.E and/or Se deficient animals, studies on purification and characterization of GSTs expressed in liver and lung tissues were undertaken in the second chapter. In the third chapter, studies on the physiological role of GSTs with special reference to their role in oxidative stress and arachidonic acid metabolism were undertaken.

METHODOLOGY

2.1.0.0. Materials

Arachidonic acid, PGD_2, PGE_2, $PGF_{2\alpha}$, GSH, GSSG, vitamin E (d-α-tocopherol), NADPH, TBA (thiobarbituric acid), Alkaline phosphatase, Freund's complete and incomplete adjuants, NBT (nitroblue tetrazolium), BCIP (5-bromo-4-chloro-3-indoyl phosphate), BHT (butylated hydroxytoulene) and DAN (2,3-diaminonaphthalene) were purchased from Sigma Chemicals Company, St. Louis, USA. ^3H arachidonic acid was prepared on request by BARC, Bombay, India. TLC plates were purchased from Merck, Germany. Ion exchangers (CM-cellulose, DE 52 cellulose) were obtained from Whatman England Inc. HPLC grade acetonitrile, methanol. acetic acid, trifluoroacetic acid, triethylamine were purchased from SD fine Chemicals India Ltd., and Spectrochem, India Ltd. All other chemicals which have not been mentioned were procured from local chemical companies and were of high quality grade.

2.2.0.0. Animals and Dietary treatment

Wistar strain female albino rats at weanling stage weighing approximately 40 g were divided into 4 groups of twelve each. The basic diet (without vitamin E and selenium) (Table 3) was supplemented with 0.5 mg Se/Kg diet, as sodium selenite, and/or with 100 IU vitamin E/Kg diet, as d α-tocopheryl acetate to create the following dietary groups: -E+Se, +E-Se, -E-Se and +E+Se. The rats were housed in individual cages, given distilled water *ad libitum* and maintained on the diet for 13 weeks. Weight gained by the animals was monitored weekly.

After 13 weeks on the experimental diets, animals were anaesthetized with ether and subjected to a whole body perfiision with a chilled 0.85% NaCl solution to remove the circulating blood from the tissues. Lung and liver tissues were excised and rinsed with ice-cold deionized water to remove any residual blood and stored at -80° C until used. Only female rats were employed in the present studies to avoid the sex differences if any in the parameters studied.

Table 3: Composition of the vitamin E and Se deficient diet

COMPONENT	AMOUNT %
Vitamin free casein	20
Cellulose	5
Sucrose	63.7
Mineral mixture*	4
Vitamin mixture**	1
Choline chloride	1
DL-Methionine	0.3
Vitamin free corn oil	5

* Selenium added as sodium selenite (0.5 mg/Kg diet) for the supplemented diets.

•• Vitamin E added as tocopheryl acetate (100 IU/Kg diet) for the supplemented diets.

2.3.0.0. Estimation of vitamin E

Vitamin E levels in liver and lung tissues were analyzed by HPLC as per the method described by Arun Sharma and Ajay Kumar, 1990 using D a-tocopheryl acetate as an internal standard.

2.3.1.0. Sample preparation

200 mg tissue was taken and homogenized in 1 mL of ethanoL To this homogenate 10 mg of internal standard was added. The sample was vortexed for about 1 min and 1 mL of n-Hexane was added and the mixture was again vortexed for 1 min. The sample was centrifuged for 5 min at 3000 rpm and the hexane layer was separated. This process of extraction was repeated again and the hexane pool was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 100 µL of methanol:diethyl ether (75:25 v/v).

2.3.2.0. HPLC separation of vitamin E

50 µL of tissue vitamin extract was injected onto the Shimpak CLC-ODS (0.45 X 25 cm) column and separated on Shimadzu LC 6AD HPLC system with methanol as the solvent at 1 mL/min flow rate. The eluant was monitored either on UV/VIS detector (297 nm) or fluorescence detector (Ex 295 nm, Em 340 nm). d-Alpha tocopheryl acetate was used as the internal standard. Tissue vit.E levels were calculated based on HPLC integration data of the sample and standard. A typical chromatogram of vitamin E separation on HPLC was presented in Fig. 11.

2.4.0.0. Estimation of selenium

Selenium was estimated according to the method of Alfthan (1984). Lung/liver tissue sample (100 mg), standards and blanks were taken into separate test tubes. The test tubes were covered with aluminium foils and few antibumping granules were added. To these tubes 0.4 mL of 1:20 sulfuric:perchloric acid (v/v) was added as digestion mixture, which results in complete recovery of resistant

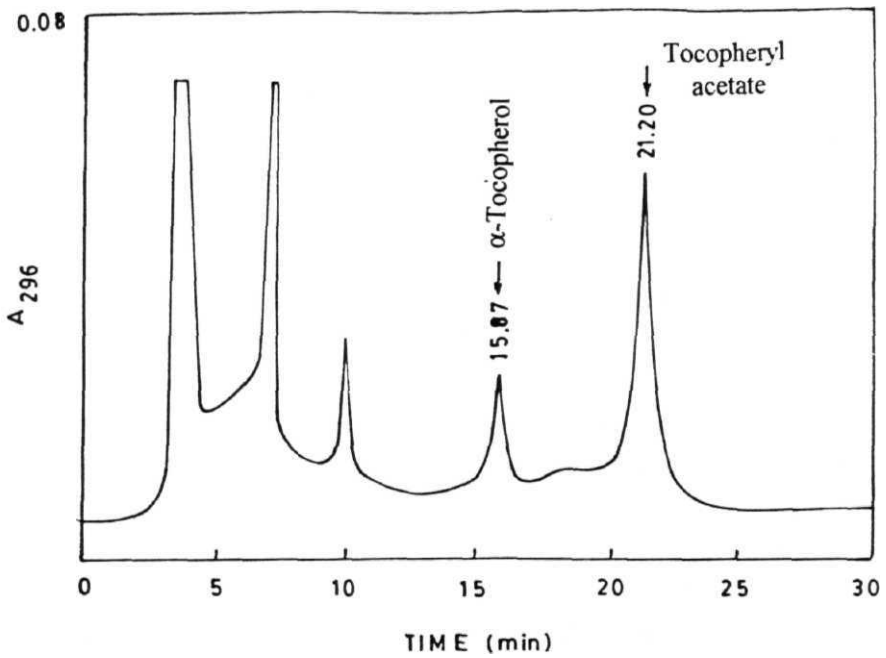


Fig. 11: Separation of vitamin E on RP-HPLC

Column.	ODS, μ Bondapak (Waters)
Solvents:	Methanol
Flow rate:	1 mL/min
Detection:	296 nm

selenium species present in biological samples. The tubes were transferred to a heating block at ambient temperature in a fume hood and then the temperature of the block was raised to 120°C, slowly over a period of 40 min and the block was maintained at the same temperature for 20 min. Nitric acid (0.5 mL) was added to prevent charring of the sample. The block temperature was next set to 150° C for 1 hr followed by 180° C for 1.5 hr. The cessation of boiling and the evolution of perchloric acid fumes were taken as signs of complete digestion, which occurred between 60 to 90 min. At the end of heating period the digest was colorless.

The test tubes were cooled and a few drops of 30% hydrogen peroxide were added to each tube and heated for 10 min at 150° C. This step was repeated because fumes of nitrogen dioxide were observed. To each cooled tube 1 mL of 6 N HC1 was added and the tubes heated at 110° C for 10 min. The tubes were removed from the heating block, 1 mL of 6 M formic acid and 1.5 mL of EDTA reagent were added to each tube and the contents were mixed well. The pH was adjusted to 1.5-2.0 with 4 M ammonia. Hereafter the tubes were protected from direct light by wrapping them with aluminium foil. To these tubes a 1 mL of 0.1% DAN reagent was added, mixed well and were placed in a water bath at 50° C for 30 min. The tubes were cooled briefly in cold water to facilitate better separation of phases, and then 2.5 mL cyclohexane was added. The tubes were stoppered and extracted vigorously, by shaking for 30 sec. The cyclohexane layer was transferred to 1-cm cuvette with a pasteur pipette and the fluorescence was measured at excitation and absorption wavelengths of 369 and 518 nm respectively using cyclohexane as blank in a fluorescence spectrophotometer.

2.5.0.0. Estimation of malondialdehyde (MDA)

Lipid peroxidation was measured, as MDA levels, via TBA reaction using HPLC (Bird *et al*,1983). 350 mg of tissue was homogenized in 0.5 mL of 1.15% KC1 and 0.5 mM BHA (in methanol) was added. To the sample 3 mL of 0.44 mol of H_3PO_4/L was added. After 10 min 0.042 mM TBA was added. The contents

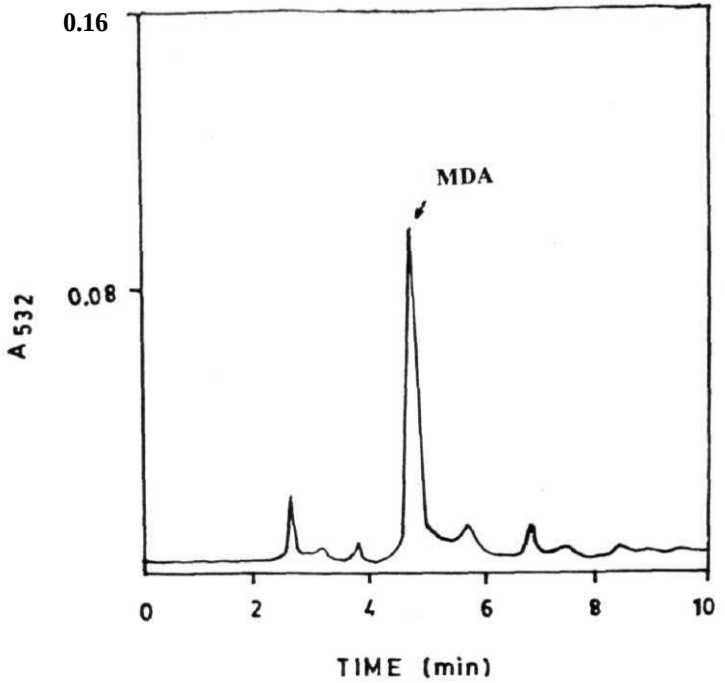


Fig. 12: RP-HPLC analysis of MDA

Column: ODS, μ Bondapak (Waters)
Solvents: Methanol: Water: Acetic acid (65:35:0.1) pH 5.7
Flow rate: 1 mL/min
Detection: 280 nm

were heated for 30 min at 90° C and after cooling 10 µL of the sample was loaded onto RP-HPLC.

The sample was loaded onto C_{18} µ Bondapak (3.9 X 300 mm) column and eluted with 50 mM KH_2PO_4, pH 7.0:methanol (65:35) at 1 mL/min flow rate. The eluant was monitored at 532 nm (Fig. 12). The trimethoxy pentane was used as external standard. Basing on HPLC integration data and on comparison with the standard value, the concentration of MDA was calculated and expressed as nmol of MDA/g wet tissue.

2.6.0.0. Antioxidant defense systems

2.6.1.0. Assay of glutathione peroxidases

Perfused tissues were homogenized (20% w/v) in 25 mM Tris-HCl; pH 7.4, containing 1 mM EDTA, 2 mM GSH and 250 mM sucrose. The homogenate was centrifuged at 10,000 X g for 30 min. The activity was measured by monitoring the oxidation of NADPH at 340 nm in a reaction mixture containing 250 mM phosphate buffer; pH 7.0, 2.5 mM EDTA, 2.5 mM sodium azide, 1 mM GSH, 0.2 mM NADPH and enzyme, incubated for 5 min at room temperature. Total GSH Px activity was assayed by using 1.5 mM cumene hydroperoxide to initiate the reaction. The Se-GSH Px activity was assayed by using 0.25 mM hydrogen peroxide to initiate the reaction. Non-Se-GSH Px activity was calculated by subtracting Se-GSH Px activity from the total peroxidase activity (Reddy *et al.*, 1981). One unit of activity was defined as one nmole of NADPH oxidized per min.

2.6.2.0. Estimation of GSH/GSSG

GSH/GSSG in tissue samples was measured by a slightly modified HPLC method (Jayatilleke and Shaw, 1993). All animals were anesthetized with anesthetic ether prior to operation for tissue perfusion. Following anesthesia, the

whole tissue was perfused *in situ* via the portal vein with iced KCl to remove blood.

The perfused tissue (~ 1 g) was homogenized at 0° C for 30 s in 4 vol of iced 1.15% KCL The homogenate was then immediately diluted 1:1 with 4% sulfo salicylic acid. Just prior to deproteinization, p-aminobenzoyl glutamate (Paba Glu) was added as an internal standard. The acidified samples were spun at 20,000 X g for 10 min and passed through a Sep-Pak Cartridge (waters) previously conditioned with 2 volumes of elution buffer. The filtrate was then analyzed for GSH/GSSG.

Samples were usually diluted in elution buffer 1:10 prior to loading into HPLC. Dilutions of 1:5 were used to measure GSSG in samples. After injecting, the compounds were eluted isocratically at 1 mL/min using 2.5 mM sodium phosphate buffer, pH 3.5; containing 0.005 M PIC-A (tetrabutylammonium phosphate) and 10% methanol and analyzed on 30 cm X 3.9 mm C_{18} u Bondapak column. Peaks were detected by UV absorbance using a Shimadzu SPD-6AV UV-VIS detector at a wavelength of 190 nm. As shown in Fig. 13, areas were corrected for recoveries to the Paba Glu added as an internal standard. The column was regenerated by washing for 15 min with 50% methanol followed by 30 min of equilibration with elution buffer.

2.6.3.0. Assay of γ-glutamylcysteine synthetase

γ-Glutamylcysteine synthetase activity was determined by the procedure described by Igarashi *et al* (1982). The standard assay reaction mixture (1.0 mL) contained 100 mM Tris-HCl; pH 8.2, 10 mM sodium L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 5 mM disodium ATP, 2 mM EDTA, 10 mM sodium L-glutamate, 10 mM L-a-aminobutyrate, 20 mM $MgCl_2$, 5 mM disodium ATP, 2 mM EDTA, 0.02 mg BSA and enzyme. The reaction was initiated by the addition of ATP. After incubation at 37° C for 30 min, the reaction was terminated by adding 1 mL of 10% TCA and centrifuged. The inorganic

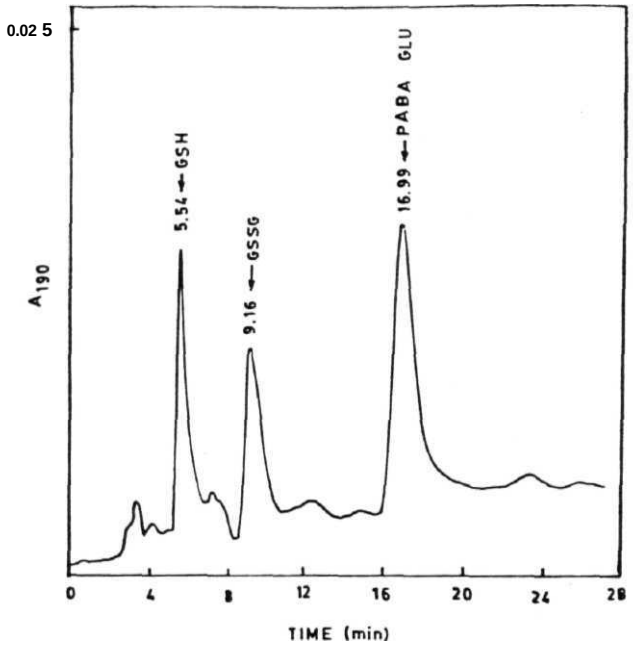


Fig. 13: Separation of GSH, GSSG and Paba Glu on HPLC

Column:	ODS, |i Bondapak (Waters)
Solvents:	13% Methanol in 25 mM NaH_2Po4; pH 3.4 containing PIC-A
Flow rate:	1 mL/min
Detection:	190 nm
Sample:	250 ng each

phosphate released was determined by the method of Fiske and Subbarow (1925). One unit of enzyme activity was defined as the amount that catalyzes the release of 1 nmole of phosphate per min.

2.6.4.0. Assay of glutathione reductase

Glutathione reductase was estimated according to the method of Carlberg and Mannervik (1985). The tissues were homogenized (20% w/v) in 10 mM phosphate buffer, pH 7.0 containing 1 mM EDTA and centrifuged at 10,000 X g for 30 min followed by 105,000 X g for 1 hr. The supernatant was used for enzyme assay. The reaction mixture in a volume of 1 mL contained 100 mM sodium phosphate; pH 7.6, 1 mM GSSG, 0.5 mM EDTA, 0.1 mM NADPH and enzyme source. The NADPH oxidation was recorded at 340 nm on UV/VIS spectrophotometer and the values expressed in units/mg protein. One unit of activity was defined as one nmole of NADPH oxidized per mm.

2.6.5.0. Assay of γ-glutamyl transpeptidase

γ-Glutamyl transpeptidase activity was assayed with L-γ-glutamyl-p-nitroanilide as substrate (Satoh *et al.,* 1980). The tissues were perfused and homogenized (20% w/v) by adding ice-chilled 1.15% KCl. The standard assay mixture contained 50 mM Tris-HCl, 20 mM glycylglycine, 10 mM $MgCl_2$, 4.4 mM L-γ-glutamyl-p-nitroanilide, pH 8.0; in a final volume of 1 mL. The reaction was initiated by the addition of protein sample and incubated at 37° C for 30 min. One unit was defined as the formation of one pmole of p-nitroanilide formed/min.

2.6.6.0. Assay of glutathione S-transferases

GST activity with CDNB was determined spectrophotometrically as described by Reddy *et al* (1983). Activities with other classical substrates were assayed as described by Habig and Jakoby (1981) (Table 4). All enzyme assays were carried out spectrophotometrically at 25° C. Blank activities were measured

Table 4: Summary of assay conditions for various GST substrates

Substrate	Buffer pH	[Substrate] mM	[GSH] mM	Absorbance nm	Δ^{ε}
1,2-Dichloro-4-nitrobenzene	7.5	1.0	5.0	345	8.5
1-Chloro-2,4-dinitrobenzene	6.5	1.0	1.0	340	9.6
Δ^5-Androstene-3,17-dione	8.5	0.068	0.1	248	16.3
1,2-Epoxy-3-(p-nitrophenoxy)- propane	6.5	0.5	5.0	360	0.5
4-Nitropyridine-N-oxide	7.0	0.2	5.0	295	7.0
p-Nitrobenzyl chloride	6.5	1.0	5.0	310	1.9
Ethacrynic acid	6.5	0.2	0.25	270	5.0
p-Nitrophenyl acetate	7.0	0.2	0.5	400	8.79
Bromosulfophthalein	7.5	0.03	5.0	330	4.5
Cumene hydroperoxide	7.0	0.03	1.0	340	6.2
Hydrogen peroxide	7.0	0.25	1.0	340	6.2
t-Butyl hydroperoxide	7.0	1.5	1.0	340	6.2
13-HODE	7.0	0.03	1.0	340	6.2
15-HPETE	7.0	0.03	1.0	340	6.2

in the absence of enzyme and subtracted from experimental values to correct for possible non-enzymatic reactions. One unit of enzyme activity was defined as one nmole of product formed or one nmole of substrate consumed per min. Specific activities were given in units/mg protein.

2.7.0.0. Purification of GSTs

All purification steps were carried at 0-4^{0}C. After each dialysis step all the samples were centrifuged at 10,000 X g for 30 min in a Kubota centrifuge to remove denatured protein. The protein concentration, GST activity and non-Se-GSH Px activity were determined at each step of purificatioa The following buffers were used during purification of GSTs from tissues. Buffer A: 22 mM potassium phosphate, pH 7.0; Buffer B: 10 mM sodium phosphate, pH 6.0; Buffer C: 10 mM Tris-HCl, pH 8.0. A typical rat tissue purification of GSTs preparation was performed as follows:

2.7.1.0. Preparation of crude extract

Female wistar strain albino rats of vit.E and/or Se supplemented and deficient dietary groups were anesthetized, liver and lung tissues were perfused and tissues were excised. The tissues were minced and homogenized in a waring blender to 20% (w/v) with buffer A containing 250 mM sucrose for 5 min and then in a porter elvejhem homogenizer with a teflon pestle. The homogenate was centrifuged at 10,000 X g for 30 min a Kubota centrifuge. The supernatant was filtered through glass wool to remove the floating lipid material and the filtrate centrifuged at 105,000 X g in a Beckman centrifuge using Ti 70 rotor. The final supernatant was passed through the glass wool/cheese cloth. The filtrate thus obtained was taken as cytosol of the tissue and referred to as the crude extract. The crude extract was dialyzed for 24 hr against 10 volumes of buffer A to remove the endogenous GSH which interferes with the binding of GSTs to the affinity column. The dialyzed tissue cytosol was applied onto GSH affinity column.

2.7.2.0. Glutathione linked epoxy activated sepharose-6B column

One gm of GSH was coupled to ten gm of epoxy activated sepharose-6B by the procedure of Simons and Vander Jagt (1977). The column was equilibrated with buffer A and the flow rate was adjusted to about 60 mL/hr. The dialyzed cytosol sample was applied onto the column and washed with the same buffer until no protein was recorded in the effluent. At that stage, the column was developed with 0.05M Tris-HCl, pH 9.6 containing 5 mM GSH. Activity appears as a single sharp peak of activity. The active fractions were concentrated and dialyzed as in the previous step.

2.7.3.0. Cation exchange chromatography on CM-cellulose column

The concentrated liver affinity purified GSTs of +E+Se animals was applied to a CM-cellulose column (2.5 X 15 cm), previously equilibrated with buffer B. The column was washed with the same buffer until the absorbance of the effluent at 280 nm was zero. The elution buffer was then changed to two linear gradients used in succession, 0-75 mM KC1 and 75-200 mM KC1 in buffer B. The first gradient was prepared from 500 mL of buffer B and the same amount of buffer B containing 75 mM KCL The second one was prepared from 100 mL of buffer B containing 75 mM KC1 and 100 mL of buffer B containing 200 mM KC1.

The active GST flow through fractions were referred to as the anionic peak. The sum of transferase activity in all fractions was calculated to estimate the total GST activity in each peak. The cationic peaks were pooled individually and concentrated by Centricon microconcentrator (Amicon Co, Danvers, MA) to determine the subunit composition with SDS gel electrophoresis. The anionic fractions were pooled and dialyzed against 100 volumes of buffer C for 24 hr (with 2 changes).

2.7.4.0. Anion exchange chromatography on DE 52 cellulose Column

The dialyzed anionic sample from the previous step was loaded onto a DE 52 cellulose column (2.5 X 15) previously equilibrated with buffer C. The column was washed with the same buffer until the absorbance of the effluent at 280 nm was zero. The elution buffer was then changed to a linear gradient of 0-100 mM KC1 prepared from 100 mL of buffer C and the same amount of buffer C containing 100 mM KCl.

2.7.5.0. High performance liquid chromatography

Affinity purified GSTs of liver and lung tissues from vit.E and/or Se supplemented and deficient animals were further analyzed by reverse-phase HPLC (RP-HPLC) (Ostlund *et al.,* 1987) using μ Bondapak C_{18} (3.9 X 300 mm) column with minor modifications (Veera Reddy *et al.,* 1995). The column was eluted with a gradient elution using 0.1% (v/v) trifluoroacetic acid in 35% acetonitrile (solvent A) and 0.1% (v/v) trifluoroacetic acid in 85% acetonhrile (solvent B) solvent systems. The sample was injected in 100% solvent A and eluted with a linear gradient of 0-40% solvent B over a period of 45 min (Veera Reddy *et al.,* 1995). The eluted polypeptides were monitored at 214 nm and their relative abundance was determined from integrated peak areas. The relative subunh concentration was represented in arbitrary units. The induction of every subunit was calculated from RP-HPLC data and the total protein recovered at the final step of affinity purification protocol (Derbel *et al.,* 1993; Veera Reddy *et al.,* 1995).

2.8.0.0. GST catalyzed Prostaglandin biosynthesis

2.8.1.0. Extraction of PGs

The coupled assay system (standard incubation mixture) in a total volume of 1 mL contained 150 mM Tris-HCl, pH 8.0; 2 mM EDTA; 5 mM tryptophan, 1 mM GSH, 100 μg GSTs, 5 units of PGH synthase and 100 μM arachidonic acid containing 0.1 uCi ^{14}C arachidonic acid. The reaction mixture was incubated at

30°C for 1 min prior to the addition of 1 mM hematin to initiate the reaction. After incubation for 1 min, the reaction was quenched with 6 N HC1 and reaction products were extracted twice with 3 volumes of chloroform. Organic phases were pooled and evaporated under N_2 and the residue was dissolved in a small volume of solvent system for TLC analysis.

2.8.1.1. TLC analysis of PGs

The samples were spotted on TLC plates and the solvent system used for TLC was of ethyl acetate: trimethyl pentane: acetic acid: water (110:50:20:100) by the method of Chang *et al* (1987a). For quantification, the TLC plates were sprayed with 50% sulfuric acid and then heated at 120° C for 10 min. Different compounds were identified by their Rf values with reference to the respective standards. The spots corresponding to respective prostaglandin standards on TLC plates were scraped off individually and the radioactivity was quantitated on liquid scintillation counter (Beckman model-1800). Activity levels were expressed in terms of % ratios of total PGs formed after deducting non-enzymatically formed PGs.

2.9.0.0. GST catalyzed LTC_4 Synthesis

2.9.1.0. Incubation and Extraction

The reaction mixture in a final volume of 1 mL contained 75 mM Tris-HCl (pH 8.0), 5 mM freshly prepared GSH and 100 µg of affinity purified GSTs or 25 µg of individual isozymes separated on CM-cellulose and DE 52 column chromatography. The reaction was initiated by transferring reaction mixture into LTA_4 Me (final concentration 30 µM) test tube, which was previously evaporated to dryness under N_2 gas. After 10 min of incubation the reaction was terminated by adding 200 uL of 6 N HCL The blanks without GSTs were processed separately. The reaction mixture was then passed through a C_{18} Sep-Pak cartridge (millipore) which was previously equilibrated with methanol and water. After

passing the sample through the cartridge, it was washed with 10 mL of double distilled water and air-dried. The adsorbed sample was then eluted with 2 mL of methanol each time and evaporated to dryness under nitrogen and redissolved in HPLC solvent.

2.9.2.0. HPLC analysis

GST catalyzed LTA_4 Me products were separated on HPLC (Shimadzu LC 6AD) by the method of Chang *et al* (1987b) using C_{18} column (3.9 X 300 mm) with the solvent system of methanol:water:acetic acid (65:35:0.1) (pH adjusted to 5.7 with TEM) at a flow rate of 1 mL/min. The eluant was monitored at 280 nm (SPD-6AV UV-VIS spectrophotometric detector) and identified based on the UV-VIS spectra and chromatography with standards. The LTC_4 Me formed was quantified basing on HPLC integration data and molar extinction coefficient of LTC_4 (40,000 cm^{-1} mM^{-1}).

2.10.0.0. SDS Poly acrylamide gel electrophoresis (SDS-PAGE)

Poh/acrylamide gel electrophoresis was conducted according to the method of Laemmli (1971) in a 0.1% SDS on a vertical slab system. The gels contained 12% acrylamide with a 29:1 ratio of acrylamide to N,N,N^1,N^1-methylene-bis-acrylamide.

Samples were boiled at 100^0 C for 5 min in the presence of loading dye containing SDS (0.1%), 2-mercaptoethanol (5% v/v), bromophenol blue (0.001% w/v) and glycerol (10% v/v) in 63 mM Tris-HCl, pH 6.8. The protein mixtures were then subjected to electrophoresis on 3% (w/v) polyacrylamide stacking gel to dissociate proteins into their individual polypeptide chains in 125 mM Tris-HCl, pH 6. The electrode buffer contained 25 mM Tris base, 192 mM glycine and 0.1% SDS.

After the completion of the electrophoresis the gel was fixed in 7.5% acetic acid and 50% methanol and later developed with silver nitrate (Oakley *et al.*,

1980). Standard molecular weight markers of Pharmacia were simultaneously run and the molecular weight of the interested peptide was calculated using UVP-2000 gel documentation software program.

2.11.0.0. Immunological Studies

2.11.1.0. Raising of antisera against purified proteins

Antibodies (Abs) were raised against affinity purified rat liver GSTs and their corresponding subunits; Ya (pooled Ya_1 and Ya_2), Yb (pooled Yb_1 and Yb_2) and Yc recovered from RP-HPLC. Similarly for lung tissue also antibodies were raised against lung affinity purified GSTs and Yc, Yb (pooled Yb_1 and Yb_2), Yk and Yp subunits which were recovered from RP-HPLC. An antigen preparation at a final protein concentration of 0.4 to 1.0 mg/mL was mixed with an equal volume of Freund's complete adjuvant. The suspension was emulsified thoroughly before injecting subcutaneously at 6-8 sites on the dorsal part of New Zealand white male rabbits of 3 months old. Further immunization was carried out on days 7, 14 and 21. Some of the rabbits received one more injection on day 28 in order to elicit a stronger response. One week after the completion of the immunization series, rabbits which were found to contain useful titers of antibodies against the injected protein, as determined by double diffusion tests, were bled and the sera were collected and stored at -20°C.

2.11.2.0. Western blot analysis (Immunoblotting)

Western blot analysis of rat liver and lung tissue GSTs were conducted according to the published procedure of Towbin *et al* (1979). Equal amounts of cytosolic proteins from the tissue samples were separated by SDS-PAGE and electroblotted onto nitrocellulose sheets by semi dry blotting protocol of LKB. The gels were initially soaked in buffer A consisting of 20 mM Tris-HCl and 192 mM glycine in 20% methanolic water. The separated peptides/proteins were transferred with a current of 0.8 mA/cm^2 for 4h. The nylon membrane was then

blocked in solution B consisting of 20 mM Tris-HCl, pH 7.5; 500 mM NaCl and 2% Tween-20 for 3 min. Then nylon membrane was washed with buffer C consisting of 20 mM Tris-HCl , pH 7.5; 500 mM NaCl and 0.05% Tween-20 for 10 min followed by buffer D consisting of 20 mM Tris-HCl, pH 7.5; 150 mM NaCl and 0.05% Tween-20 for 10 min X 3 times each. The nylon membrane was then treated with buffer D containing 1% non fat dried milk and the primary antibody for 1hr. The membrane was then washed thoroughly for 30 min with buffer C to remove non-specific binding of the antibody with the membrane. The membrane was then treated with buffer D containing secondary Ab (alkaline phosphatase) and allowed to bind to primary Ab for 1 hr. The nylon membrane was then washed with buffer C for 10 m X 3 times each and finally developed with buffer E consisting of 100 mM Tris-HCl; pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 4 mM NBT, 4.5 mM BCEP till the blue color appears on the membrane. The reaction was stopped by changing the membrane from buffer E to distilled water and later dried for photography.

2.12.0.0. Protein Estimation

Protein concentrations were estimated by the method of Lowry *et al* (1951) and also by the method of Warburg and Christian (1941). Blanks were subtracted where appropriate to correct for absorbance due to the interference of the buffers in the assay.

2.13.0.0. Statistical analysis

The data was analyzed with one-way analysis of variance followed by Student Newman Kuel's test. The significance was set at $P<0.05$.

CHAPTER I

3.1.0.0. INTRODUCTION

Molecular oxygen is an essential nutrient for higher forms of life. In addition to its normal physiological reactions, oxygen and its partially reduced forms, ROS oxidize a variety of macromolecular and simpler compounds in cells and fluids of the body. When ROS production is excessive, such as during prolonged aerobic exercise, or when antioxidant defense is severely hampered by nutritional deficiencies, or pharmacological intervention, an inadequate defense may be overwhelmed by the ROS, leading to extensive cell and tissue damage (Esterbauer *et al,* 1991).

When PUFAs on the biomembranes are attacked by free radicals in the presence of molecular oxygen, a chain of peroxidative reactions occur, eventually leading to the formation of hydrocarbon gases and aldehydes (eg; malondialdehyde). Byproducts of lipid peroxidation are the most frequently studied markers of oxidative tissue damage. As a protection against excessive oxidation, nature has developed a complex set of interactive antioxidant systems. Biological antioxidants include SOD, catalase, γ-glutamyl cycle enzymes like Se-GSH Px and glutathione reductase. Nonenzymatic biological antioxidants include tocopherols, ascorbic acid and glutathione. Nutritional deficiency provides an excellent model to study the dynamic balance between oxidative challenge and antioxidant defense in the biological system. Among the nutritional deficiencies, vit.E and Se form the most important dietary factors contributing to tissue antioxidant defenses.

Vitamin E functions as an important cellular antioxidant in the hydrophobic compartments, protecting PUFAs from lipid peroxidation (Burton and Ingold, 1989; Kagan *et al,* 1989). Consequently dietary vit.E deprivation and/or exercise may result in increased lipid peroxidation products such as MDA and possible disruption of membrane function (Sjodin *et al,* 1990; Tiidus *et al.,* 1993). Selenium has been recognized as an essential trace element in animal nutrition playing a protective role against peroxidative damage in the animal cells, mainly in

the form of GSH Px (Sunde and Hoekstra, 1980). Se deficiency is known to result in the loss of GSH Px activity, the enzymes involved in the reduction of hydroperoxides (Hill *et al,* 1987; Ji *et al.,* 1988). The role of vit.E and Se in the prevention of coronary heart disease (Van-Poppel *et al.,* 1994; Luoma *et al.,* 1995), muscle damage and soreness (Kanter, 1994), cystic fibrosis (Kauf *et ah,* 1995) and cancer (Schwartz *et al.,* 1994) has been extensively studied. Although liver is equipped with abundant antioxidant enzymes and other scavenging systems, the high metabolic rate and central role in detoxification make it one of the main targets for free radical damage in the body, moreso under impaired antioxidant defenses. Animals fed on diet deficient in vit.E and/or Se nutrients develop a number of pathological conditions (Scott, 1978). In addition to respiratory function, the lung offers a first line of defense against environmental toxicants, pollutants and oxidants. Lung is the principal target organ for oxygen poisoning in mammals. Severe morphological and functional alterations in the lung are caused by hyperoxic conditions, often resulting in death (Crapo *et al.,* 1980b). Selenium deficiency has been shown to augment the pulmonary toxic effects of oxygen exposure in the rat (Hawker *et al.,* 1993).

A stable intracellular redox state is of vital importance to organisms (Krentzchmar and Klinger, 1990). Many enzymes require the essential thiols to be kept in reduced state. As the most important non-protein thiol source, GSH concentration in the cell is remarkably high. Liver is the major organ for *de novo* GSH synthesis and supplies 90% of the circulating GSH (Potter and Tran, 1993). From the studies in mice and rats, liver has been recognized as the central organ of the interorgan homeostasis of GSH, serving as the principal source of GSH for plasma (Lauterburg *et* al., 1984). Lung, like liver and kidney shows a high rate of GSH turnover (Martensson *et al.,* 1989). Studies in isolated perfused rat lung indicate use of plasma GSH by the lung. Thus GSH and associated enzyme systems play an important role in mediating cellular antioxidant defenses. Liver and lung are the main tissues vulnerable to oxidative damage in animals subjected to oxidative stress. Hence in the present study the rate of lipid peroxidation and

the metabolism of glutathione in liver and lung tissues were analyzed in animals fed on vit.E and Se deficient diets in order to assess the oxidative stress induced in relation to the animals fed on vit.E and Se supplemented diets.

3.2.0.0. RESULTS

Wistar strain female albino rats at weanling stage were divided into four groups of 12 animals each and they were fed on -E+Se, +E-Se, -E-Se and +E+Se diets for a period of 13 weeks. After the dietary treatment period, animals were killed by cervical dislocation. Liver and lung tissues were excised after perfusion with physiological saline and kept in -80°C until used for biochemical analysis.

3.2.1.0. Vitamin E

Feeding vit.E deficient (-E+Se, -E-Se) diet for a period of 13 weeks to wistar strain female albino rats, starting from the weanling stage, resulted in a significant decrease in vit.E levels both in liver and lung tissues when compared to supplemented animals (+E-Se and +E+Se) (Fig. 14A&B). Vitamin E levels in these tissues fell to approximately 10% of levels of +E+Se animals.

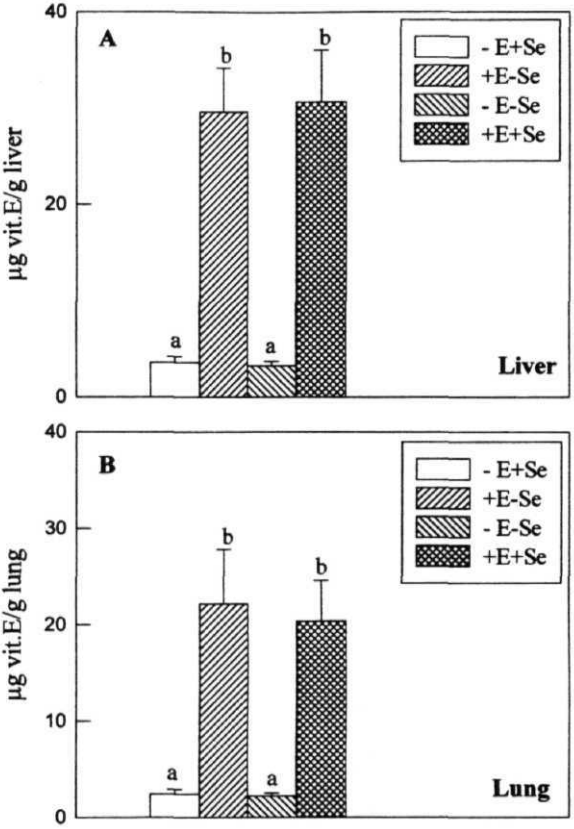
3.2.1.2. Selenium

Similarly feeding animals with Se deficient diets (+E-Se and -E-Se) resulted in significant depletion of Se both in lung and liver tissues (Fig. 15A&B). Selenium levels in the tissues of Se deficient animals fell to 5% of levels in Se supplemented animals.

3.2.1.3. Growth and tissue somatic indices

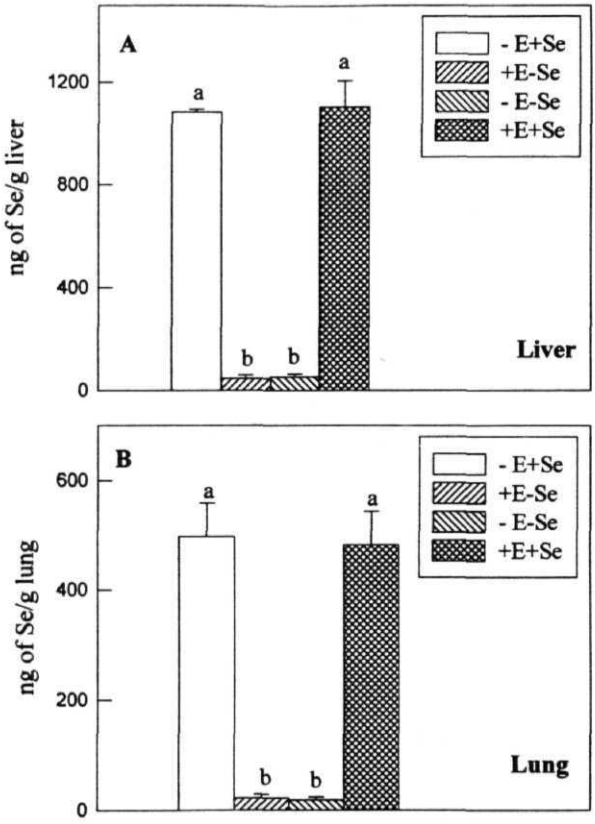
The dietary treatment given for a period of 13 weeks starting from weanling stage resulted in significant changes in the growth pattern (Fig. 16) and tissue somatic indices (Fig. 17A&B). Animals fed on vit.E and Se supplemented diets (+E+Se) maintained the highest growth pattern compared to all other groups,

Fig. 14: Vitamin E levels in liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets for 13 weeks



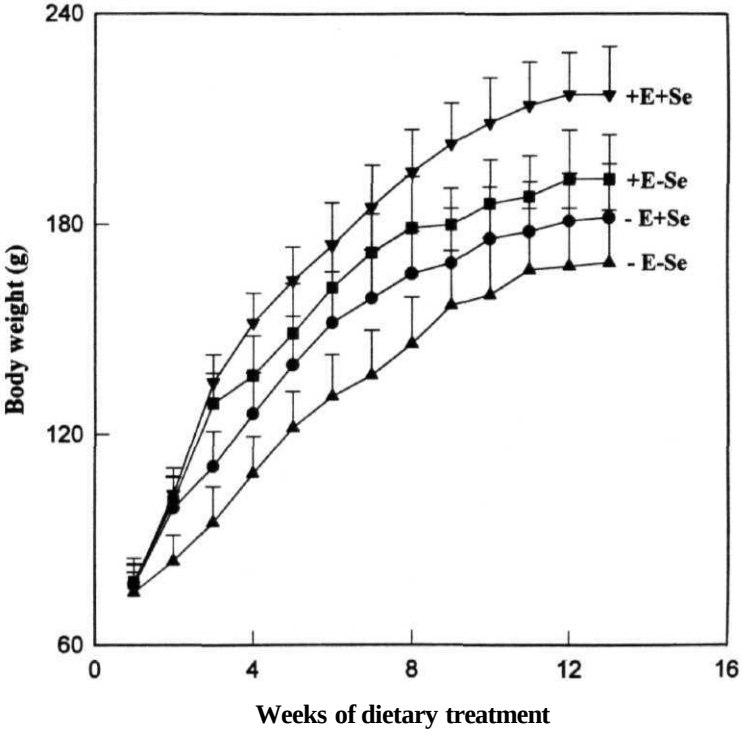
Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test.
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 15: Selenium levels in liver and lung tissues of rats fed on vit.E and/or Se supplemented anddeficient diets



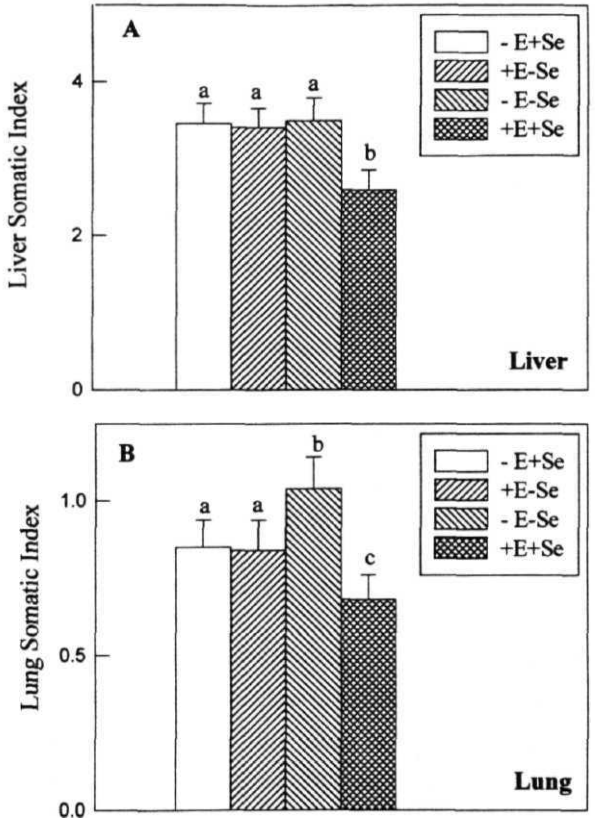
Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 16: Growth curves of rats fed on vit.E **and/or Se supplemented and deficient diets**



Each value is the mean + SD of 12 individual observations.

Fig. 17: Tissue somatic indices of rats fed on vit.E and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test.
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

throughout the period of dietary treatment. Animals fed on -E-Se diets, on the other hand, showed the reduced growth rate compared to all the other groups studied. The other two groups (+E-Se and -E+Se) showed intermediate growth rate between +E+Se and -E-Se groups. Tissue somatic indices (TSI) of both liver and lung tissues were significantly lower in the +E+Se group of rats compared to all other groups (Fig. 17A&B).

3.2.1.4. Lipid Peroxidation

RP-HPLC analysis was used to separate lipid peroxides in the form of MDA adduct from other chromogens absorbing at 532 nm. Use of HPLC eliminates artiiacts due to the reaction of TBA with other body-fluid constituents to give different chromogens. As shown in Fig. 18A, significantly higher concentrations of MDA were observed in liver tissue of -E+Se (32%), +E-Se (106%) and -E-Se (136%) group of animals when compared to supplemented (+E+Se) animals. Similarly in lung tissue also higher levels of MDA were observed in -E+Se (35%), +E-Se (161%) and -E-Se (200%) animals when compared to supplemented animals (Fig. 18B).

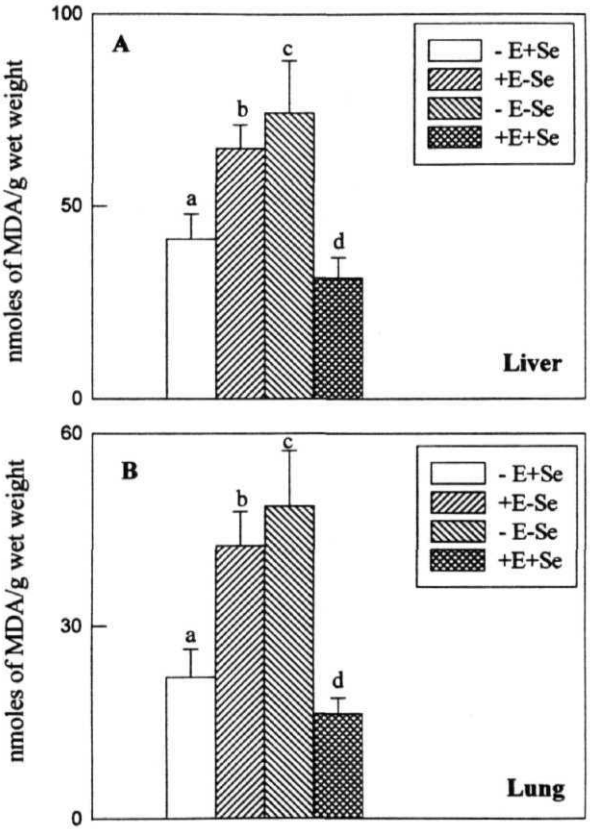
3.2.2.0. Antioxidant defense systems

The levels of cytosolic antioxidant enzymes like Se-glutathione peroxidase, γ-glutamylcysteine synthetase, glutathione reductase, γ-glutamyl transpeptidase and glutathione S-transferases were measured in liver and lung tissues of rats fed on the different diets. The levels of GSH and GSSG were determined in perfused tissues immediately after isolation.

3.2.2.1. Se-glutathione peroxidase

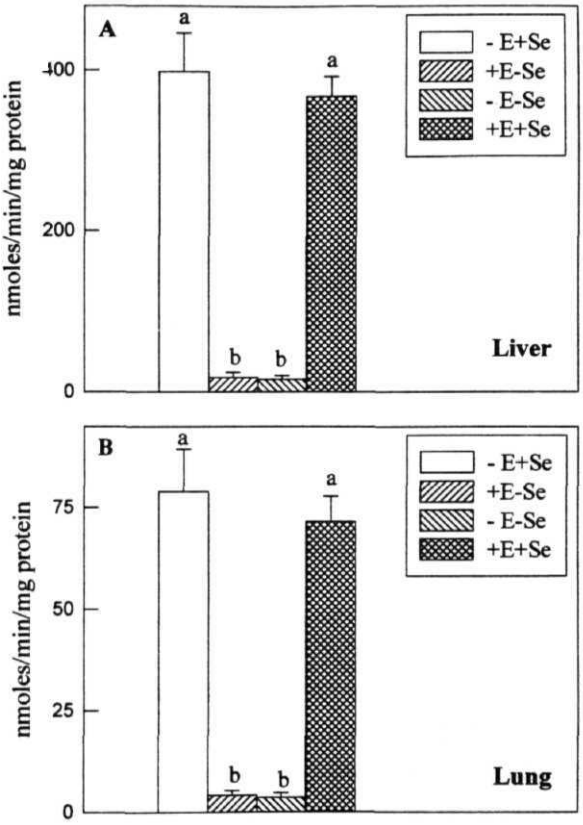
Se-GSH Px, the enzyme involved in the reduction of inorganic and organic hydroperoxides, activity levels reached to insignificant levels in liver and lung tissues of Se deficient animals (+E-Se and -E-Se) compared to the Se supplemented ones (-E+Se and +E+Se) (Fig. 19A&B). This observation suggests

Fig. 18: Lipid Peroxidation in liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test.
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 19: Activity levels of Se-GSH Px with H_2O_2 in the cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test.
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

the efficiency of dietary treatment in depleting tissue Se levels and thereby decreasing the activity levels of Se-dependent enzymes.

3.2.2.2. Reduced glutathione

The levels of both GSH and GSSG were determined simultaneously on RP-HPLC. In liver tissue GSH levels were significantly higher in +E-Se (66 %) and -E-Se (83.4%) animals when compared to +E+Se animals. In contrast, GSH levels in lung tissue were significantly lower in -E+Se (-24%), +E-Se (-41.8%) and -E-Se (-55.22%) animals when compared to +E+Se animals (Fig. 20A&B).

3.2.2.3. Oxidized glutathione

In liver and lung tissues the levels of GSH were 5 fold higher than GSSG levels. GSSG levels in liver of Se deficient animals were significantly lower (-45% in +E-Se and -45.7% in -E-Se) when compared to Se supplemented (-E+Se and +E+Se) animals (Fig. 21 A). However in lung tissue GSSG levels were significantly higher in -E+Se (70%), +E-Se (181%) and -E-Se (216%) animals when compared to that of +E+Se animals (Fig. 21B).

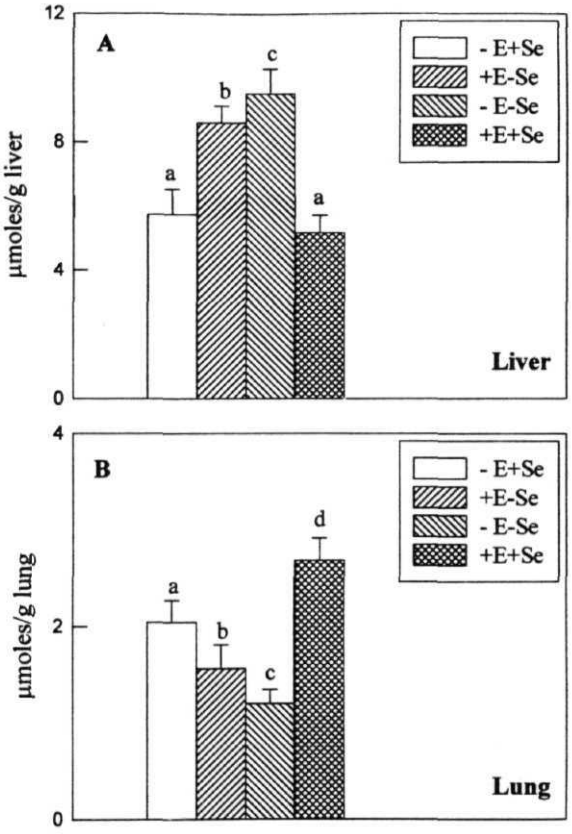
3.2.2.4. γ-Glutamylcysteine synthetase

Glutamylcysteine synthetase activity levels in both liver and lung tissues of -E+Se, +E-Se, and -E-Se animals were significantly higher compared to +E+Se groups (Fig. 22A&B). In liver tissue GCS levels were significantly higher in -E+Se (70%), +E-Se (109%) and -E-Se (153%) group of animals. Similarly, in lung tissue higher levels of GCS were observed in -E+Se (40%), +E-Se (197%) and -E-Se (210%) groups when compared to +E+Se animals.

3.2.2.5. Glutathione reductase

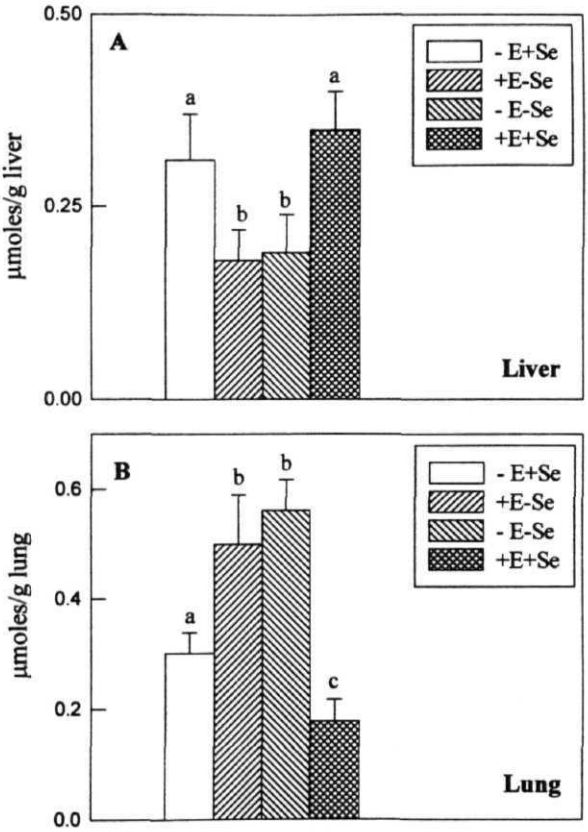
Glutathione reductase activities in the present study were increased in the liver and lung of all deficient rats when compared to supplemented animals. The

Fig. 20: Levels of GSH in liver and lung tissues of rats fed on vit.F. and/or Se supplemented and deficient diets



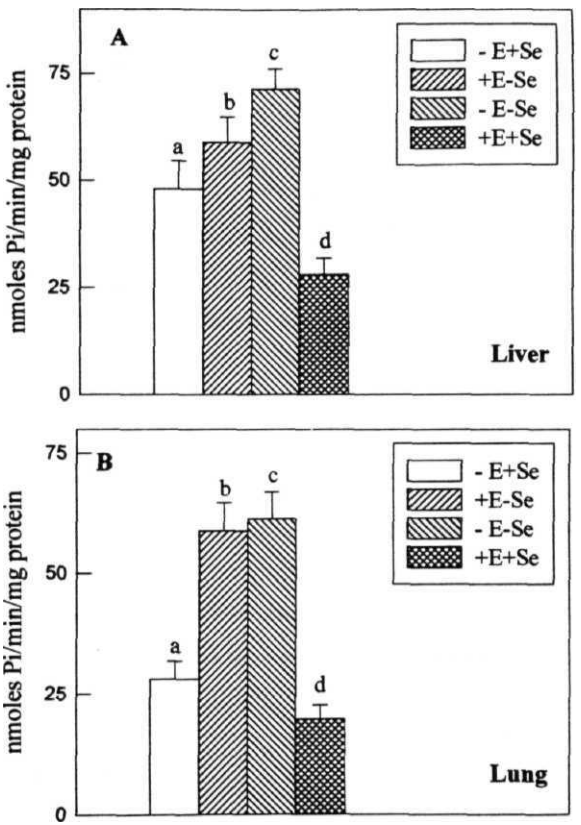
Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test.
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 21: Levels of GSSG in liver and lung tissues of rats fed on vit £ and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test.
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 22: Activity levels of γ-glutamylcysteine synthetase in the cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

hepatic glutathione reductase activities were significantly higher in -E+Se (46%), +E-Se (61%) and -E-Se (81%) animals when compared to that of +E+Se animals (Fig. 23A). Similarly in lung tissue, all deficient groups showed significantly higher levels (40% in -E+Se, 63% in +E-Se and 81% in -E-Se) compared to +E+Se animals (Fig. 23B).

3.2.2.6. γ-Glutamyl transpeptidase

Liver, in spite of the highest concentration of GSH showed lesser GGT activity compared to lung tissue. GGT activity levels were significantly reduced in liver tissue of vit.E and/or Se deficient animals when compared to +E+Se animals (Fig. 24A). In contrast, GGT activity levels in the lung tissue of vit.E and/or Se deficient animals were significantly higher compared to that of +E+Se animals (Fig. 24B).

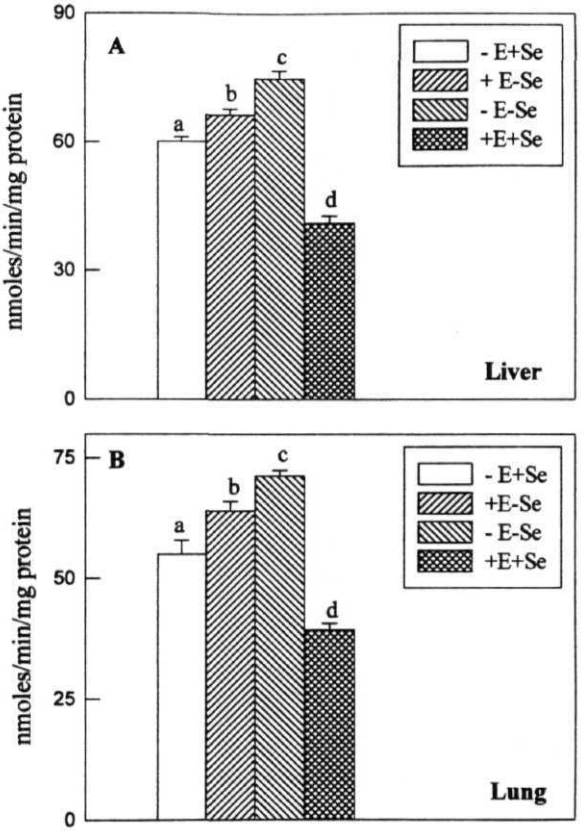
3.2.2.7. Glutathione S-transferases

The hepatic GST activities were significantly higher in +E-Se (48.5%) and -E-Se (77%) animals when compared to that of +E+Se animals (Fig. 25A). However in lung tissue all deficient groups showed significantly higher levels (48% in -E+Se, 40% in +E-Se and 80% in -E-Se) compared to +E+Se animals (Fig. 25B).

3.3.0.0. Discussion

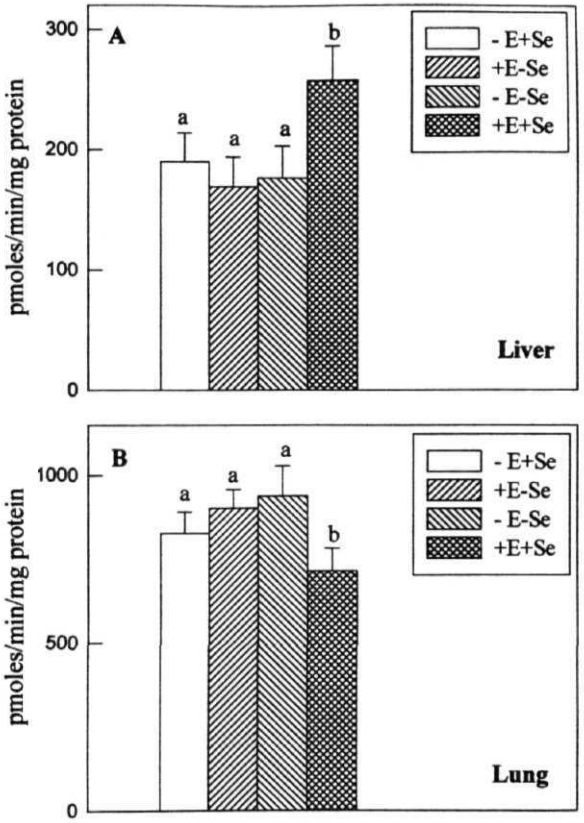
Vit.E, as a chain breaking free radical scavenger, protects the tissue from non-enzymatic lipid peroxidation. Vh.E has been shown to be involved in the inhibition of LOX (Reddanna *et al.*, 1989, Lomnitski *et al.*, 1991) and in the reduction of hydroperoxides catalyzed by LOX (Cucurou *et al.*, 1991). Thus vit.E plays an important antioxidant role both against enzymatic and non-enzymatic lipid peroxidations. Selenium, as an integral component of Se-GSH Px, plays an important role in the reduction of organic and inorganic hydroperoxides.

Fig. 23: Activity levels of glutathione reductase in the cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



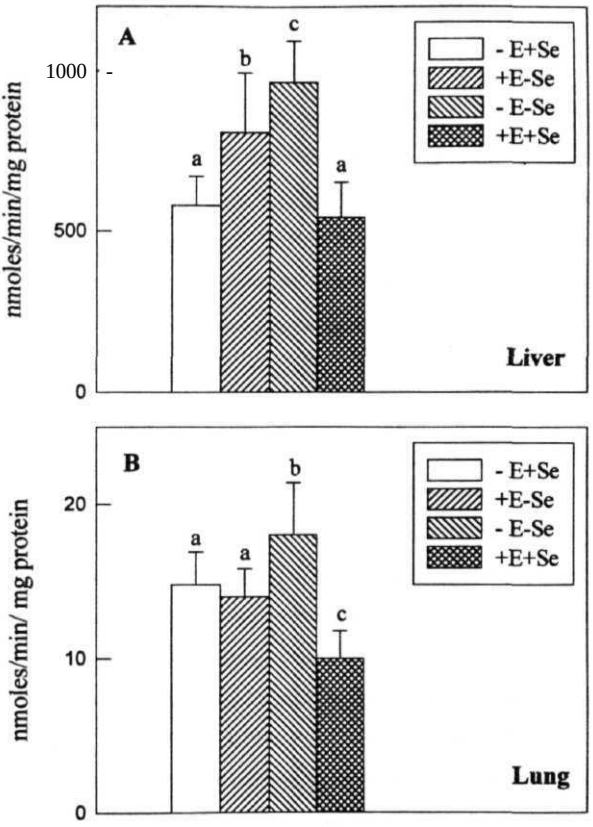
Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test.
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 24 : Activity levels of γ-glutamyl transpeptidase in the cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 25: Activity levels of GSTs with CDNB in the cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test.
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

In view of their key role in tissue antioxidant defenses, vit.E and/or Se deficiency markedly reduced the growth rate of female albino rats (Fig. 16). As a result of this liver and lung tissue somatic indices also got affected (Fig. 17). The increase in the TSI of liver and lung tissues in vit.E and/or Se deficient animals in comparison to the supplemented group, is more due to the decrease in the body weights rather than increase in tissue weights.

The impaired antioxidant defenses as a result of induced vit.E and Se deficiency could be responsible for the observed increase in lipid peroxides in animals deficient in Se and/or vit.E (Fig. 18A). *In vivo,* MDA is formed almost exclusively from peroxidation of PUFAs, hence its presence is thought to be a prime indicator of the occurrence of tissue lipid peroxidation (Gutteridge and Halliwell, 1990). Similar increase in lipid peroxides was reported in liver, lung and cardiac muscles of animals fed on vit.E and/or Se deficient diets (Hafeman and Hoekstra, 1977; Tiidus *et al.*, 1993; Awad *et ah,* 1994; Rokizki *et ah,* 1994). The present study also has clearly demonstrated that liver and lung tissues of vit.E and/or Se deficient animals are vulnerable to impaired antioxidant defenses. Se-GSH Px and other Seleno peroxidases play a prominent role in the reduction of both inorganic and organic hydroperoxides in different compartments of cells. Selenium deficiency in the present study has resulted in significant depletion of Se-GSH Px activity levels (Fig. 19A). Similarly other Seleno peroxidases such as plasma GSH Px and phospholipid hydroperoxidase might have been reduced in Se deficient animals. In addition deficiency of vit.E also might be responsible for the observed increase in the levels of lipid peroxides in both liver and lung tissues of vit.E and/or Se deficient animals (Fig. 18A&B).

In the present study Se deficiency resulted in increased liver GSH levels (Fig. 20A). No significant differences, however, were observed in liver tissue of vit.E deficient animals (Fig. 20A). Hill and Burk (1982) have studied GSH metabolism in isolated rat hepatocytes and demonstrated that GSH synthesis and turnover are accelerated significantly by Se deficiency and not significantly by vit.E

deficiency. This implies that Se deficiency but not of vit.E accelerates GSH synthesis in rat liver. These changes were associated with increased activities of rate-limiting enzymes in the GSH turnover such as GCS in vit.E and/or Se deficient animals (Fig. 22A). Also glutathione reductase, another enzyme involved in GSH turnover, was also at higher levels in liver of vit.E and Se deficient animals (Fig. 23A). This increased glutathione reductase activity might be a mechanism for increasing GSH status to protect liver from the prevailing oxidant stress in nutrient deficient animals. The increased levels of GSH in Se deficient animals could be due to increased demand for GSH caused by increased tissue lipid peroxides.

GGT is an important component of the y-glutamyl cycle in cells (Meister and Anderson, 1983). The γ-glutamyl bond can be cleaved by GGT, an enzyme located on the external surface of cell membranes of various tissues. Liver is known to have negligible GGT activity (Bray and Taylor, 1993). In the present study also GGT activity in liver was much lower compared to lung tissue (Fig. 24A). GGT activity levels in liver were further decreased in vit.E and Se deficient livers when compared to that of +E+Se animals. Hepatic uptake of plasma GSH is very low as a result of the relatively low levels of GGT activity in the liver (Lauterburg *et ed.,* 1984). GSH as well as other γ-glutamyl-containing compounds, including GSSG and y-glutamyl glutathione, react with GGT at the outer cell surface. The y-glutamyl moiety is transferred to a suitable amino acid acceptor, and both the y-glutamyl amino acid and cysteinylglycine are transported into the cell and reused for GCS and glutathione reductase reactions to enhance cellular GSH levels. Although the first step in GSH degradation is catalyzed by GGT, it seems unlikely that hepatic GGT is involved in the reaction in the liver, because of its low level and its further decrease by vit.E and Se depletions. Therefore, GSH release from liver is of primary importance in the GSH turnover. Perhaps, GSH and/or GSSG released into the blood are cleaved and reabsorbed in kidney and the lung tissues, containing very high activity levels of GGT and other related enzymes. In relation to this point, Hill and Burk (1982) observed that Se deficiency raised plasma GSH plus GSSG level but not cysteine levels in rats.

In addition to increased GSH levels, vit.E and/or Se deficiency has resulted in significantly higher activity levels of GST in liver tissue (Fig.25A). This observation is consistent with the results of Chang *et al.,* (1990) and Christensen *et al.,* (1994) where elevated GSTs were reported in response to Se deficiency. GSTs, are multifunctional proteins known mainly for detoxification of xenobiotics. The induction of GSTs in response to vit.E and Se deficiency indicates their possible role in antioxidant defenses also. GSTs of a family are known for their role in the reduction of organic peroxides (Chang *et al.,* 1990). It will be interesting to probe further on the specific isozymes of GSTs induced in response to nutritional deficiency and their involvement in antioxidant defenses.

Antioxidants and glutathione related enzymes play a significant role in the defense responses of the lung tissue to oxidant stress (Panus *et al.*, 1988). Se-GSH Px activities were decreased in Se deficient tissues (Fig. 19B) which was also evidenced by studies of Forman *et al* (1983) and Jenkinson *et al* (1989). Se deficient lung has reduced activity of Se-GSH Px and, hence, limited capacity to metabolize H_2O_2 and other peroxides. GSH exported from liver to the blood plasma is utilized by the lung, which like liver and kidney exhibits a high overall rate of GSH turnover. In the present study, Se and vit.E deficiency increased GGT activity of lung tissue (Fig. 24B). Activated GGT of lung tissue facilitates the import of substrates required for GSH synthesis. Exercise training is known to increase GGT activities of lung and muscles in rat, but decrease in liver (Sen *et al.,* 1992). During deficiency when hepatic efflux of GSH is accelerated, the lung tissue enjoys a greater ability to enrich its GSH-dependent antioxidant and detoxicant status.

Se and/or vit.E deficiency in the present study enhanced GCS activity of lung tissue (Fig. 22B). This effect was more pronounced in -E-Se animal tissues, which should result in higher GSH levels. On the contrary, GSH levels decreased in lung tissues of -E-Se animals (Fig. 20B). Lungs from Se deficient rats were shown to release glutathione in response to oxidative stress (Jenkinson *et al.*,

1987). Mice treated with buthionine sulfoxamine (BSO) reduce total lung GSH levels and thereby increasing the susceptibility to oxygen-induced lung damage (Smith and Anderson, 1992). Several lung disorders are believed to be characterized by an increase in alveolar oxidant burden, potentially depleting GSH levels in lung tissue (Martensson *et al.*, 1989). Low GSH has been linked to abnormalities in the lung surfactant system and the interaction between GSH and antiproteases in the epithelial lining fluid of patients. In new born rats and in adult mice GSH deficiency results in extensive damage to lung type 2 cells with decreased number of lamellar bodies and decreased amounts of intraalveolar tubular myelin (Martensson *et ah,* 1989, 1991). Thus when there is a marked decrease in cellular GSH, the normal physiological formation of reactive oxygen species is unopposed, and this leads to severe cellular damage.

The decreased GSH during vit.E and/or Se deficiency might be due to increased GSH utilization by enzymes such as GSTs, which are induced (Fig. 25B). The rate of depletion by these electrophilic agents is related to the level of GST activity in the cell. Thus GSSG levels were increased which is an indication of oxidative stress. The relatively high concentrations of GSSG may be due to concomitant increase in pulmonary GSTs and increased levels of peroxides in deficient tissues (Fig.22B). Jaeckson and Veal (1990) and Reuter and Klinger (1992) found a marked increase in GSSG levels of lung tissue after hypoxia and reoxygenation. Thus if the rate of GSH consumption exceeds that of import, a tissue may show a net deficit of GSH. The glutathione reductase activities were higher in the lung tissue of vit.E and/or Se deficient animals compared to that of supplemented animals, more pronounced in -E-Se lung tissue to recycle GSH (Fig.23B). Glutathione reductase levels, however, were lower in animals supplied with vit.E and Se compared to deficient animals, indicating the secondary role played by thiols in antioxidant defense mechanisms of lung tissue in the presence of vit.E and Se. Thus it is clear that vit.E and Se deficiency results in inducing oxidant stress in the lung tissue, which is being tackled mainly by cellular thiols. In the present study the observed increase in the levels of GST activities in nutrient

deficient animals suggests a compensatory role for glutathione in protecting hepatic and pulmonary tissues from the oxidative damage induced by vit.E and/or Se deficiency (Fig. 25B).

The present study therefore, reveals that lung tissue is relatively more susceptible to oxidative damage induced by vit.E and/or Se deficiency. As a result of induced oxidative stress, liver and lung tissues of vit.E and/or Se deficient animals showed induction of GSTs. Further studies, however, are required to identify the specific isozyme(s) of GST involved in antioxidant defenses.

CHAPTER II

4.1.0.0. INTRODUCTION

In the first chapter it is demonstrated that either vit.E and/or Se deficiency induces oxidative stress in female albino rats. Vitamin E and/or Se deficiency also induced GSTs in lung and liver tissues significantly. In order to identify the isozymes of GSTs induced, GSTs were purified by affinity chromatography and further separated on SDS-PAGE. The subunit composition of GSTs, however, was analyzed on RP-HPLC.

Earlier differences in tissue distribution and expression of GST isozymes have been documented mainly by comparison of SDS-PAGE and immunoblotting (Tu *et al.,* 1983; Hayes and Mantle, 1986). However, there were and still are problems with cross-reactivity of GST antibodies and quantification by immunoblotting. Development of an HPLC technique by Ostlund *et al* (1987) significantly improved the ability to identify and quantify GST proteins, and has been used extensively (Ketterer *et al.,* 1988, Hayes *et al.,* 1990). In the present study the HPLC technique is modified so that all known subunits can be separated with essentially baseline resolution.

4.2.0.0. RESULTS

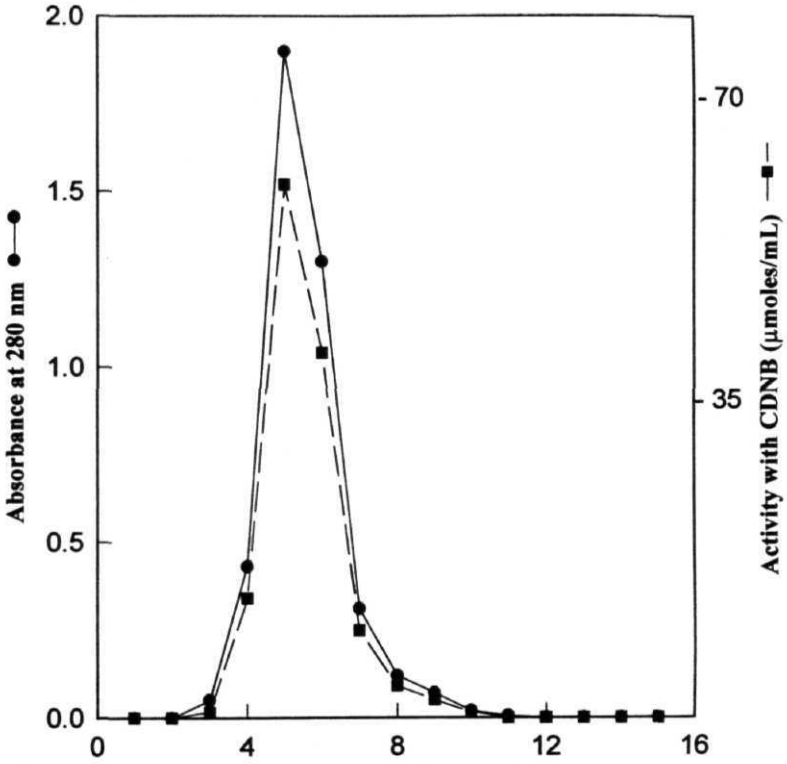
Female wistar strain albino rats at weanling stage were fed with -E+Se, +E-Se, -E-Se and +E+Se diets for a period of 13 weeks. After the dietary regimen, animals were killed and liver and lung tissues excised after perfusion. Cytosolic GSTs from tissues were purified by GSH linked agarose column chromatography. Fig. 26 represents the typical chromatogram of affinity purification of rat liver and/or lung cytosolic GSTs.

4.2.1.0. Hepatic GSTs

4.2.1.1. Affinity chromatography

Purification of GSTs by GSH-affinity chromatography was carried out five

Fig. 26: Affinity purification of rat liver and/or lung cytosolic GSTs



times separately for each group using pooled liver tissue (12 g) from respective groups. Table 5 to 8 shows the typical purification profiles of GSTs from liver tissues of -E+Se, +E-Se, -E-Se and +E+Se animals. As shown in the profiles, increase in specific activity was observed in liver crude extracts (10,000 X g) of +E-Se (49%) and -E-Se (77%) animals when compared to that of +E+Se animals. Similarly the specific activity levels in the cytosolic fractions (105,000 X g supernatant) of +E-Se and -E-Se animals also were much higher compared to +E+Se animals. As a result, the overall purification fold of +E-Se (33%) and -E-Se (43%) animals decreased when compared to -E+Se and +E+Se animals. This observed increase in the specific activity levels in the crude extracts as well as cytosolic fractions and decrease in the final fold purification are indicative of induction of GSTs in the liver tissue of +E-Se and -E-Se animals. Also the total affinity purified GST protein levels were much higher in +E-Se (50%) and -E-Se (77%) animals than the levels found in +E+Se animals. No appreciable differences in specific activity (37 U/mg protein) and % yield (~70%) were observed in the affinity purified liver GSTs of animals fed on vit.E and/or Se supplemented and deficient diets. The comparison of total activity, total protein and yield at the final step of liver GST purification for all the dietary groups is represented in table 9.

4.2.1.2. SDS-PAGE and western blot analysis of GSTs from cytosolic extracts

The affinity purified liver GSTs were resolved into 3 subunits on SDS-PAGE (Fig. 27, lane 1&6) with molecular weights of 25.6, 27 & 28 kDa, which were designated as Ya, Yb and Yc respectively, as per the nomenclature of Mannervik *et al* (1985). In order to determine whether increased GST activity in +E-Se and -E-Se was due to an increased amount of GST protein, equal amounts of liver cytosol protein (20 µg) was loaded and separated on SDS-PAGE (Fig. 27). Western blotting analysis was performed after electrophoresis using polyclonal antibodies raised against affinity purified liver GSTs (Fig. 28A), rat liver Yc (Fig. 28B), rat liver Yb (Fig. 28C) and rat liver Ya subunits (Fig. 28D).

TABLE 5: PURIFICATION PROFILE OF LIVER GSTs OF RATS FED ON -E+Se DIET

Step	Total activity units (U)	Total protein (mg)	Specific activity (U/mg)	Yield %	Purification fold
10,000 X g	837.5	2417	0.346	100	1
105,000 Xg	743.0	1279	0.581	88.73	1.68
Affinity pooled	563.6	15.4	36.6	67.3	105.8

Each value is the mean of five individual observations.
One unit is defined as one umole of thioether formed/min.

TABLE 6: PURIFICATION PROFILE OF LIVER GSTs OF RATS FED ON +E-Se DIET

Step	Total activity units (U)	Total protein (mg)	Specific activity (U/mg)	Yield %	Purification fold
10,000 X g	1152	2458	0.469	100	1
105,000 X g	1035	1281	0.808	89.8	1.72
Affinity pooled	765.4	20.8	36.8	66.4	78.46

Each value is the mean of five individual observations.
One unit is defined as one umole of thioether fonned/min.

TABLE 7: PURIFICATION PROFILE OF LIVER GSTs OF RATS FED ON -E-Se DIET

Step	Total activity units (U)	Total protein (mg)	Specific activity (U/mg)	Yield •/.	Purification fold
10,000 Xg	1344.5	2409	0.558	100	1
105,000 X g	1222.3	1268	0.964	91	1.728
Affinity pooled	919.7	24.79	37.1	66.31	66.48

Each value is the mean of five individual observations.
One unit is defined as one umole of thioether formed/min.

TABLE 8: PURIFICATION PROFILE OF LIVER GSTs OF RATS FED ON +E+Se DIET

Step	Total activity units (U)	Total protein (mg)	Specific activity (U/mg)	Yield •/.	Purification fold
10,000 Xg	762.9	2422	0.315	100	1
105,000 Xg	693.6	1275	0.544	90.9	1.729
Affinity pooled	511.0	14	36.5	67.0	115.9

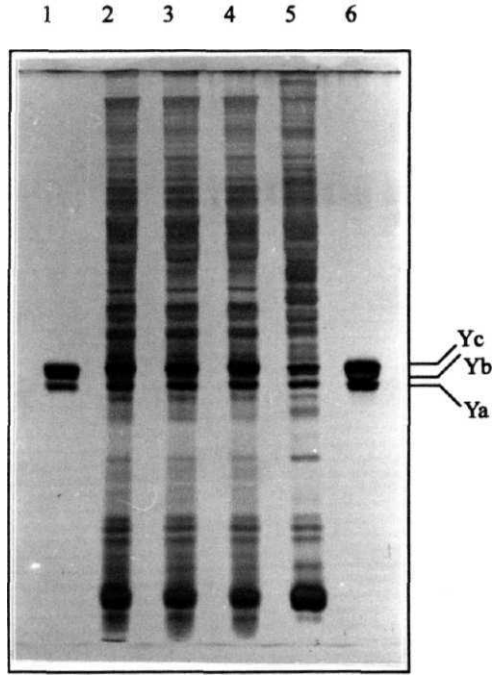
Each value is the mean of five individual observations.
One unit is defined as one umole of thioether formed/min.

TABLE 9: PURIFICATION PROFILE OF LIVER GSTs OF RATS FED ON vit.E AND/OR Se SUPPLEMENTED AND DEFICIENT DIETS

Step	Total activity units (U)	Total protein (mg)	Yield
-E+Se	564 ±89*	15.4 ±2.0*	36.6 ±2.1*
+E-Se	765 ± 110[b]	20.8 ± 3.2[b]	36.8 + 2.3[a]
-E-Se	920 ± 173[b]	24.8 ± 3.6[b]	37.0 ±2.9*
+E+Se	511 ±93*	14.0 ±2.3*	36.5 ± 2.8*

Each value is the mean ± SD of five individual observations. One unit is defined as one μmole of thioether formed/min. The same alphabet denotes no significant difference between the two groups in each column. Significance was set at $p < 0.05$.

Fig. 27: SDS-PAGE of liver cytosolic proteins of rats fed on vit.E and/or Se supplemented and deficient diets



Lane:
1. 1 |ig liver affinity purified GSTs
2. 20 µg -E+Se liver cytosolic proteins
3. 20 µg +E-Se liver cytosolic proteins
4. 20 µg -E-Se liver cytosolic proteins
5. 20 µg +E+Se liver cytosolic proteins
6. 2 µg liver affinity purified GSTs

Fig. 28: Immunoblots of liver cytosol and affinity purified GSTs of rats fed on vit.E **and/or Se supplemented and deficient diets separated on SDS-PAGE and probed with antibodies raised against**

A. Rat liver affinity purified GSTs B. Rat liver GST Yc subunit C. Rat liver GST Yb subunit D. Rat liver GST Ya subnnit

Blot A to D

Lane 1. 1 μg rat liver affinity purified GSTs

Lane 2. 20 μg -E+Se rat liver cytosolic proteins

Lane 3. 20 μg +E-Se rat liver cytosolic proteins

Lane 4. 20 ng -E-Se rat liver cytosolic proteins

Lane 5. 20 μg +E+Se rat liver cytosolic proteins

Lane 6. 2 μg rat liver affinity purified GSTs

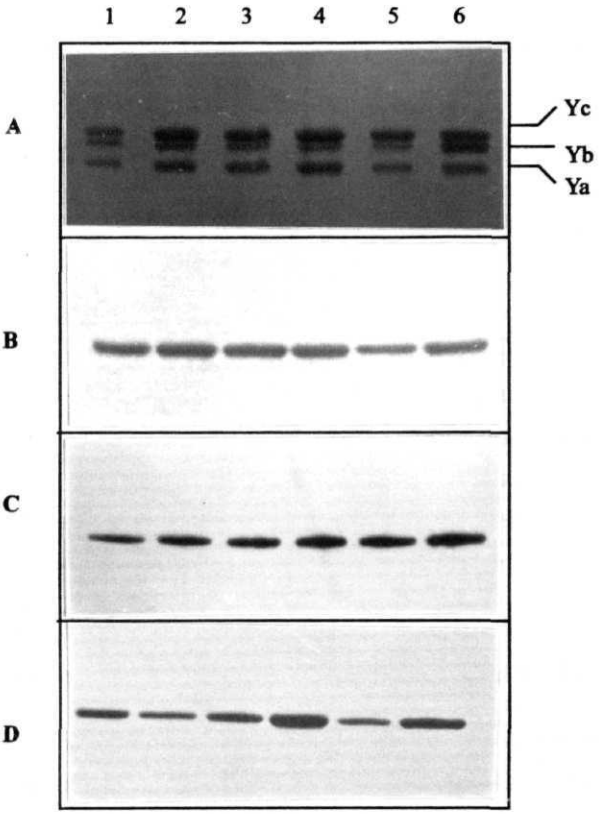

1
2
3
4
5
6
A
B
C
D
Yc
Yb
Ya

Figure 28A shows a clear induction of Yc and Ya subunits in -E+Se, +E-Se and -E-Se animals when cytosolic proteins separated on SDS-PAGE were probed with anti-affinity purified GST antibodies (compare lane 2,3,4 to lane 5). Similar results were observed when the proteins were probed with GST Yc antibody (Fig. 28B). Western blot analysis with GST Ya antibodies further snowed the induction of Ya subunit in -E+Se, +E-Se and -E-Se animals, more so in -E-Se animals when compared to that of +E+Se animals (Fig. 28D). No significant changes were observed in Yb subunit (Fig. 28A&C).

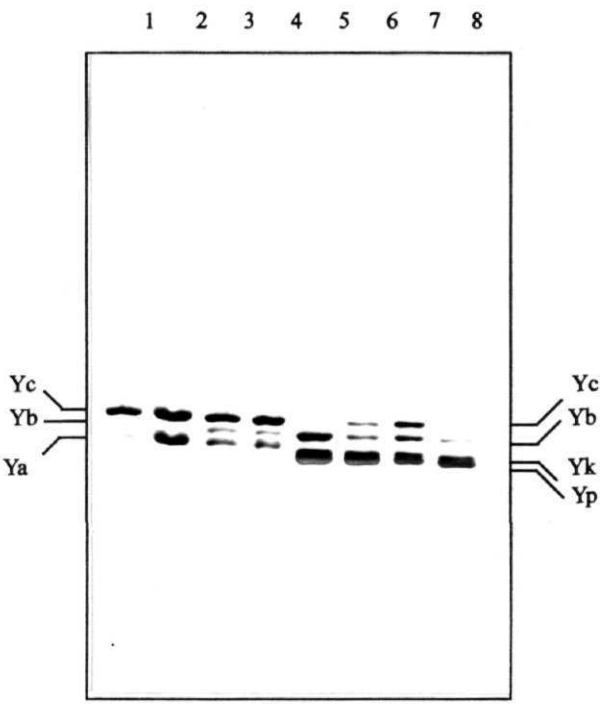
4.2.1.3. SDS-PAGE analysis of affinity purified GSTs

In order to further characterize the GSTs, equal amounts (3 µg each) of affinity purified GSTs from vit.E and/or Se supplemented and deficient animals were separated on SDS-PAGE (Fig. 29). The relative concentration of subunits were analyzed by measuring subunit concentration on UVP-gel documentation system and also from the total protein recovered at the final step of purification (Fig. 30). The relative concentration of Ya subunit was significantly higher in liver tissues of -E+Se (34%), +E-Se (61%) and -E-Se (164%) animals compared to that of +E+Se animals. Further Yc subunit was also induced in -E+Se (24%), +E-Se (57%) and -E-Se (62%) animals when compared to that of +E+Se animals (compare lanes 2,3&4 with 1 in Fig. 30).

4.2.1.4. Reverse-phase HPLC analysis of affinity purified GSTs

In order to further quantitate the specific induction of GST subunits during vit.E and/or Se deficiency, equal amounts of affinity purified GSTs were separated on reverse-phase HPLC (Fig. 31A,B,C&D). Individual peaks obtained were identified based on their order of elution as per the profile given by Johnson *et al* (1992) and were confirmed by comparison of HPLC profiles of individual isozymes and SDS-PAGE analysis. When relative subunit concentrations were compared, Ya_2 was the major Ya-sized subunit in the rat liver, which was significantly higher in -E+Se (1.43 fold), +E-Se (2.42 fold) and -E-Se (3 fold)

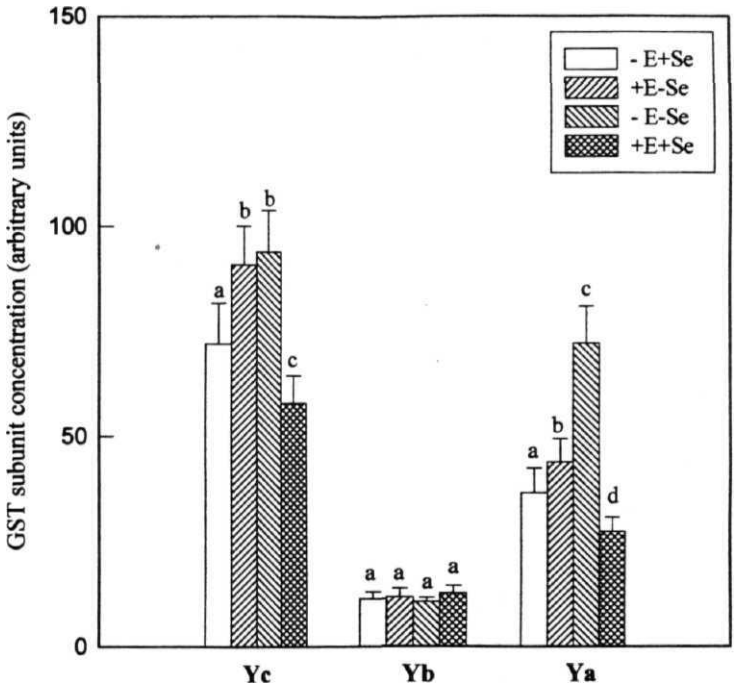
Fig. 29: SDS-PAGE of liver and lung affinity purified GSTs of rats fed on vitE and/or Se supplemented and deficient diets



Lane 1 to 4 corresponds to liver GST proteins (3 ug each) purified from
1. +E+Se 2. -E-Se 3. +E-Se 4. -E+Se animals

Lane 5 to 8 corresponds to lung GST proteins (3 μg each) purified from
5. +E+Se 6. -E-Se 7. +E-Se 8. -E+Se animals

Fig. 30: Relative subunit concentration of liver affinity purified GSTs of rats fed on vit.E and/or Se supplemented and deficient diets separated on SDS-PAGE



Values represent mean + SD of five individual experiments. Data was analyzed with a one way ANOVA followed by SNK test Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 31: RP-HPLC analysis of affinity purified liver GSTs of (A)-E+Se (B)+E-Se (C)-E-Se (D) +E+Se rats

Equal protein (100 μg) was loaded to RP-HPLC

Column: ODS (Waters)
Solvent: A. 35% Acetonitrile + 0.1% TFA
B. 85% Acetonitrile + 0.1% TFA
Flow rate: 1 mL/min
Detection: 214 nm

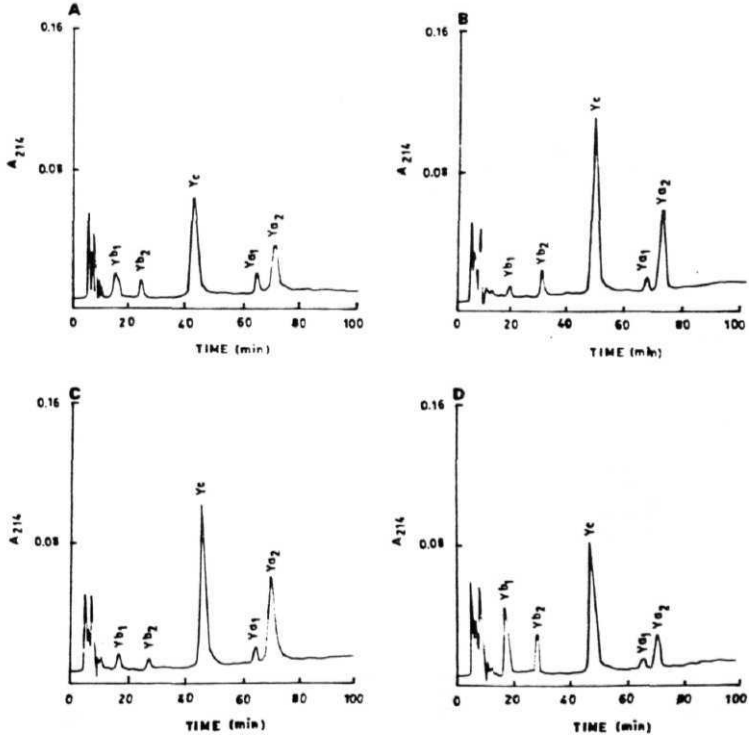
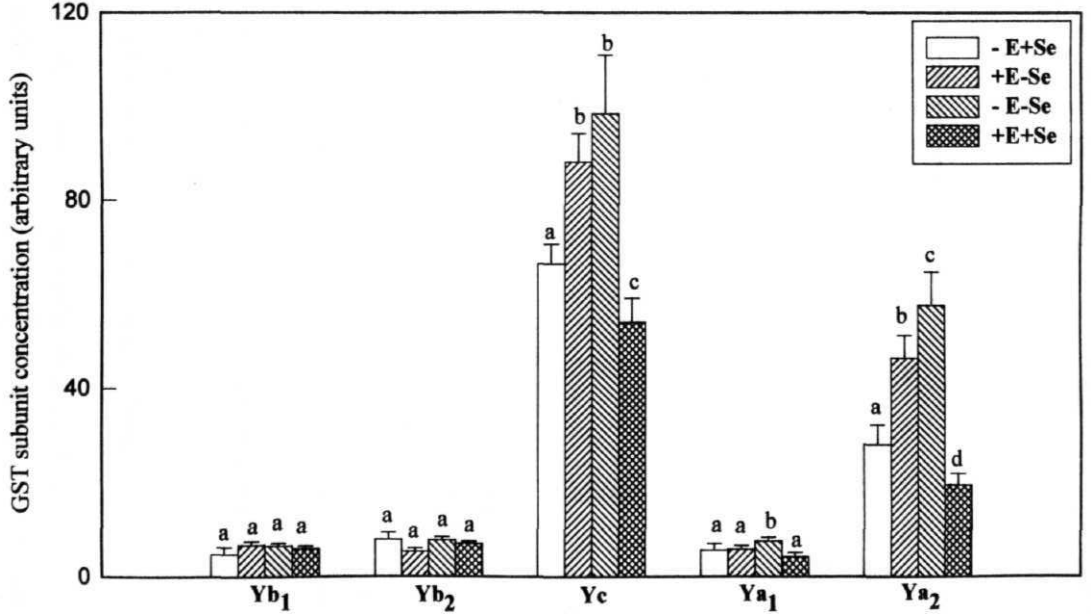


Fig. 32: Relative subunit concentration of liver affinity purified GSTs of rats fed on vit.E and/or Se supplemented and deficient diets separated on RP-HPLC



Bars represent mean ± SD of five individual experiments. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another ($p \leq 0.05$).

animals (Fig. 32). Similarly Yc was also induced in all deficient animals by 1.2 fold in -E+Se, 1.63 fold in +E-Se, and 1.82 fold in -E-Se animals compared to that of +E+Se animals. Ya_1 subunit was induced only in -E-Se (1.78 fold) animals (Fig. 32C).

4.2.2.0. Lung GSTs

4.2.2.1. Affinity chromatography

Like liver GSTs, lung GSTs were also purified from equal quantities (16 g) of pooled tissue from vit.E and/or Se deficient and supplemented animals by affinity chromatography for 3 times. The data was presented in tables 10, 11, 12 & 13. Comparison of specific activities during the first step of purification (crude extract of 10,000 X g supernatant) indicated increase in the specific activities in -E+Se (33%), +E-Se (64%) and -E-Se (76%) animals compared to the level observed in +E+Se animals. The purification fold was decreased by 16% in -E+Se, 40.12% in +E-Se and 39.13% in -E-Se animals. Further total GST protein obtained after affinity purification in lung tissues of -E+Se (48.5%), +E-Se (93%), and -E-Se (98%) animals were significantly higher when compared to the protein levels obtained for +E+Se animals. All these observations demonstrate the induction of lung GSTs in vit.E and/or Se deficient animal*; No significant differences were observed in specific activity (17 U/mg protein) and % yield (~90%) of affinity purified lung GSTs of vit.E and/or Se supplemented and deficient diets. Comparison of total activity, total protein and yield of affinity purified lung GSTs for the vit.E and/or Se supplemented and deficient diets was presented in table 14.

4.2.2.2. Western blot analysis of cytosolic extracts

The affinity purified GSTs were resolved into 4 subunits on SDS-PAGE with molecular weights of 24, 25, 27 and 28 kDa, which were designated as Yp, Yk, Yb and Yc respectively, based on their molecular weights (Fig. 33). The subunit induction was analyzed by separating equal amounts (40 µg each) of

TABLE 10: PURIFICATION PROFILE OF LUNG GSTs OF RATS FED ON -E+Se DIET

Step	Total activity units (U)	Total protein (mg)	Specific activity (U/mg)	Yield %	Purification fold
10,000 X g	15.56	1765	0.008	100	1
105,000 Xg	15.10	1019	0.015	97.02	1.805
Affinity pooled	14.30	0.815	17.61	92.24	2147

Each value is the mean of three individual observations.
One unit is defined as one μmole of thioether formed/min.

TABLE 11: PURIFICATION PROFILE OF LUNG GSTs OF RATS FED ON +E-Se DIET

Step	Total activity units (U)	Total protein (mg)	Specific activity (U/mg)	Yield %	Purification fold
10,000 X g	19.9	1803	0.011	100	1
105,000 X g	18.45	2025	0.014	92.68	1.27
Affinity Pooled	17.92	1.062	16.80	90.0	1527

Each value is the mean of three individual observations.
One unit is defined as one μmole of thioether formed/min.

TABLE 12: PURIFICATION PROFILE OF LUNG GSTs OF RATS FED ON -E-Se DIET

Step	Total activity units (U)	Total protein (mg)	Specific activity (U/mg)	Yield •/.	Purification fold
10,000 X g	21.75	1834	0.012	100	1
105,000 Xg	20.43	1121	0.018	93.92	1.54
Affinity pooled	18.36	1.07	17.1	90.67	1554

Each value is the mean of three individual observations.
One unit is defined as one umole of thioetber formed/min.

TABLE 13: PURIFICATION PROFILE OF LUNG GSTs OF RATS FED ON +E+Se DIET

Step	Total activity units (U)	Total protein (mg)	Specific activity (U/mg)	Yield •/.	Purification fold
10,000 X g	11.25	1677	0.007	100	1
105,000 Xg	10.35	1030	0.01	92.03	1.49
Affinity pooled	9.39	0.549	17.11	88.45	2553

Each value is the mean of three individual observations.
One unit is defined as one umole of thioether formed/min.

TABLE 14: PURIFICATION PROFILE OF LUNG GSTs OF RATS FED ON vit.E AND/OR Se SUPPLEMENTED AND DEFICIENT DIETS

Step	Total activity units (U)	Total protein (mg)	Yield
-E+Se	14.3± 1.6[a]	0.82 ± 0.08[a]	17.6 ± 1.6[a]
+E-Se	17.92± 2.0[a]	1.06± 0.12[a]	16.8 ±1.9*
-E-Se	18.36± 2.9[a]	1.07 + 0.11[a]	17.1 ± 2.0[a]
+E+Se	9.39 + 1.3[b]	0.55 ± 0.07[b]	17.1 ± 1.8[a]

Each value is the mean + SD of three individual observations. One unit is defined as one μmole of thioether formed/min. The same alphabet denotes no significant diflFerence between the two groups in each column. Significance was set at $p < 0.05$.

cytosolic proteins from -E+Se, +E-Se and -E-Se and +E+Se animals on SDS-PAGE (Fig. 33) and probing them with polyclonal antibodies raised against affinity purified lung GSTs (Fig. 34A), lung Yc (Fig. 34B), lung Yb (Fig. 34C), lung Yk (Fig. 34D) and lung Yp subunits (Fig. 34E). Comparison of lanes 2,3&4 with that in lane 5 of Fig. 34A&B clearly showed an induction of Yc subunit in vit.E and/or Se deficient animals. No significant changes, however, were observed in Yb, Yk and Yp subunits in vit.E and/or Se deficient animal tissues compared to that of supplemented animals (Fig. 34A,C,D&E).

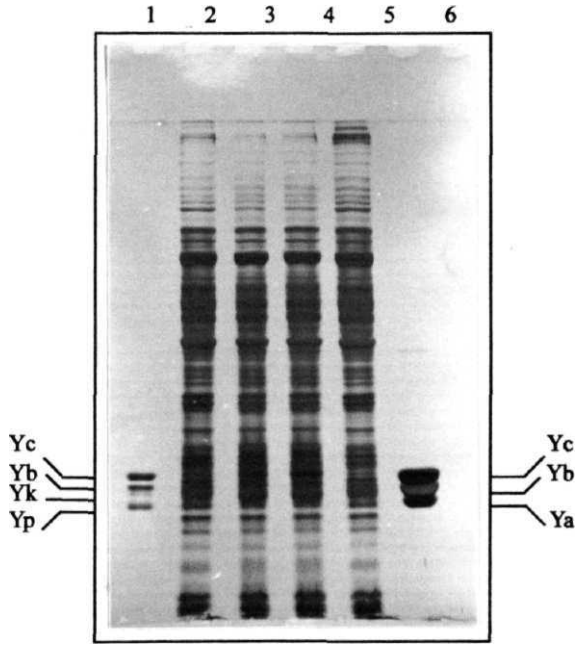
4.2.2.3. SDS-PAGE and RP-HPLC analysis of affinity purified GSTs

Equal amounts (3 μg) of affinity purified lung GSTs from vit.E and/or Se deficient and supplemented animals were separated on SDS-PAGE (Fig. 29) and relative subunit concentrations were analyzed based on UVP-gel documentation data and from the total protein recovered at the final step of affinity purification (Fig. 35). A significant induction of Yc subunit was observed in -E+Se (2.84 fold), +E-Se (4.82 fold) and -E-Se (7.48 fold) animals in comparison with +E+Se animals (compare lanes 6,7&8 with 5 in Fig. 35). No significant changes, however, were observed in Yb, Yk and Yp subunits.

In order to verify the specific induction of subunits in vit.E and/or Se deficient tissues, equal amounts (100 μg) of affinity purified GSTs were separated on RP-HPLC using Waters C_{18} analytical column (Fig. 36 A,B,C&D). When relative subunit concentrations were analyzed, a significant induction of Yc subunit was observed in -E+Se (2.9 fold), +E-Se (4.86 fold) and -E-Se (7.76 fold) animals when compared to that of +E+Se animals (Fig. 37).

Thus SDS-PAGE, immunoblots and RP-HPLC analysis of affinity purified GSTs have clearly demonstrated the induction of Ya_2 and Yc subunits in liver and Yc only in lung tissue of vit.E and/or Se deficient animals when compared to those in +E+Se animals.

Fig. 33: SDS-PAGE of lung cytosolic proteins of rats fed on vit.E and/or Se supplemented and deficient diets



Lane: 1. 1 (ig lung affinity purified GSTs
2. 40 µg -E+Se lung cytosolic proteins
3. 40 µg +E-Se lung cytosolic proteins
4. 40 µg -E-Se lung cytosolic proteins
5. 40 µg +E+Se lung cytosolic proteins
6. 3 µg liver affinity purified GSTs

Fig. 34: Immunoblots of lung cytosol and affinity purified GSTs of rats fed on vit.E and/or Se supplemented and deficient diets separated on SDS-PAGE and probed with antibodies raised against

A. **Rat** lung affinity purified GSTs **B. Rat lung** GST **Yc subunit**

C. Rat lung GST **Yb subunit** **D. Rat lung** GST **Yk subunit**

E. Rat lung GST **Yp subunit**

Blot A

Lane 1. 2 ug rat lung affinity purified GSTs

Lane 2. 40 ug -E+Se rat lung cytosolic proteins

Lane 3. 40 μg +E-Se rat lung cytosolic proteins

Lane 4. 40 μg -E-Se rat lung cytosolic proteins

Lane 5. 40 ug +E+Se rat lung cytosolic proteins

Lane 6. 1 ug rat liver affinity purified GSTs

Blot B to D

Lane 1. 2 μg rat lung affinity purified GSTs

Lane 2. 40 ug -E+Se rat lung cytosolic proteins

Lane 3. 40 μg +E-Se rat lung cytosolic proteins

Lane 4. 40 ug -E-Se rat lung cytosolic proteins

Lane 5. 40 μg +E+Se rat lung cytosolic proteins

Lane 6. 2 ug rat lung affinity purified GSTs

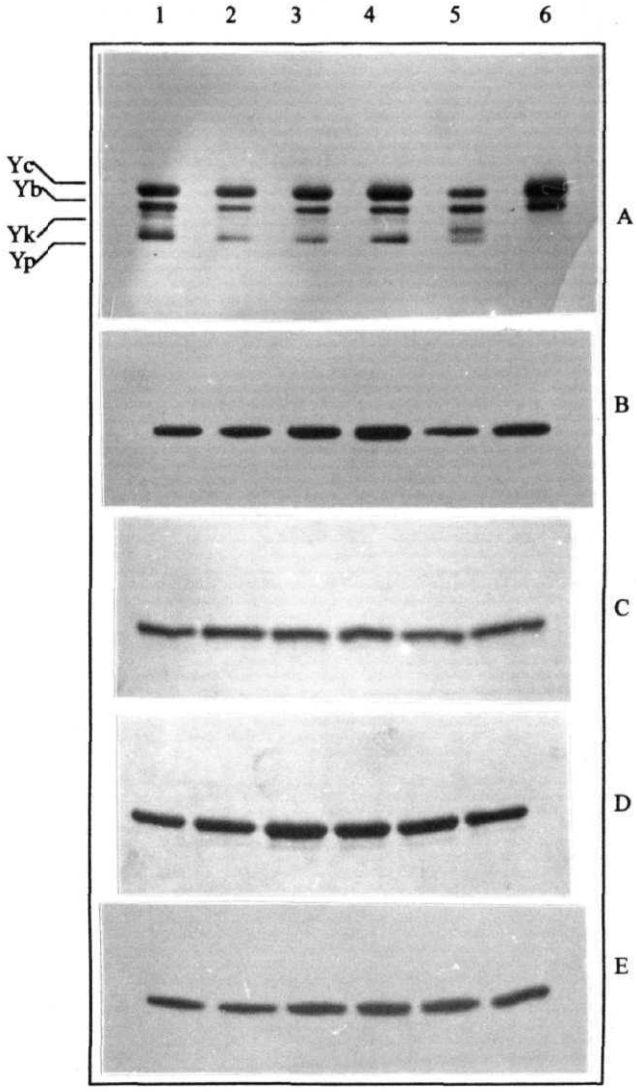
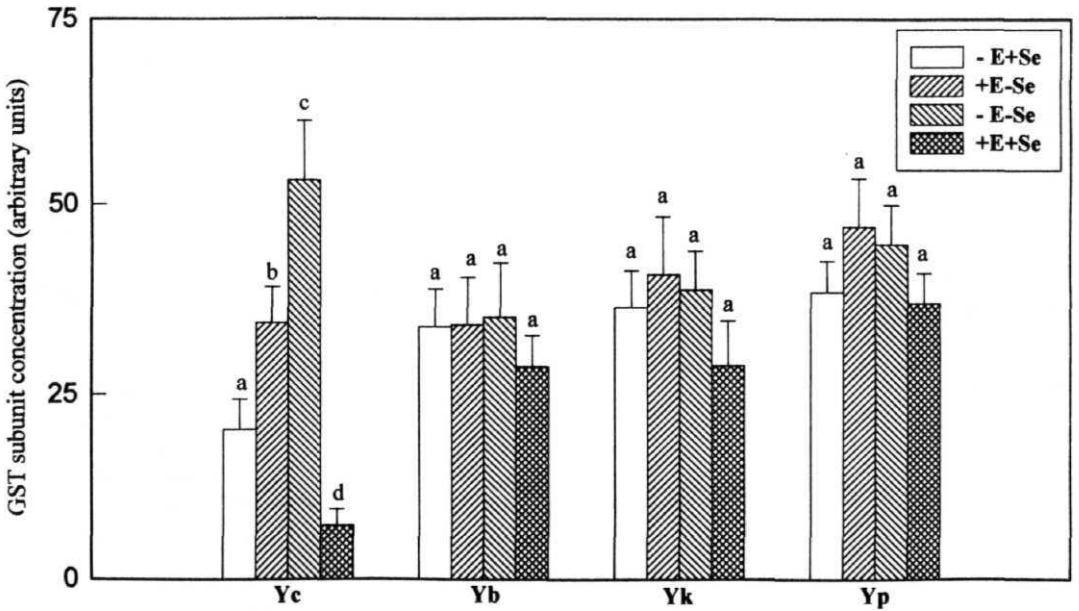

1
2
3
4
5
6
Yc
Yb
Yk
Yp
A
B
C
D
E

Fig. 35: Relative subunit concentration of lung affinity purified GSTs of rats fed on vit.E and/or Se supplemented and deficient diets separated on SDS-PAGE



Bars represent mean ± SD of three individual experiments. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another ($p \leq 0.05$).

Fig. 36: RP-HPLC analysis of affinity purified lung GSTs of (A)-E+Se (B)+E-Se (C)-E-Se (D)+E+Se rats

Equal protein **(100 µg)** was loaded to RP-HPLC

Column: ODS (Waters)
Solvent: A. 35% Acetonitrile + **0.1%** TFA
B. 85% Acetonitrile + 0.1% TFA
Flow rate: 1 **mL/min**
Detection: **214 nm**

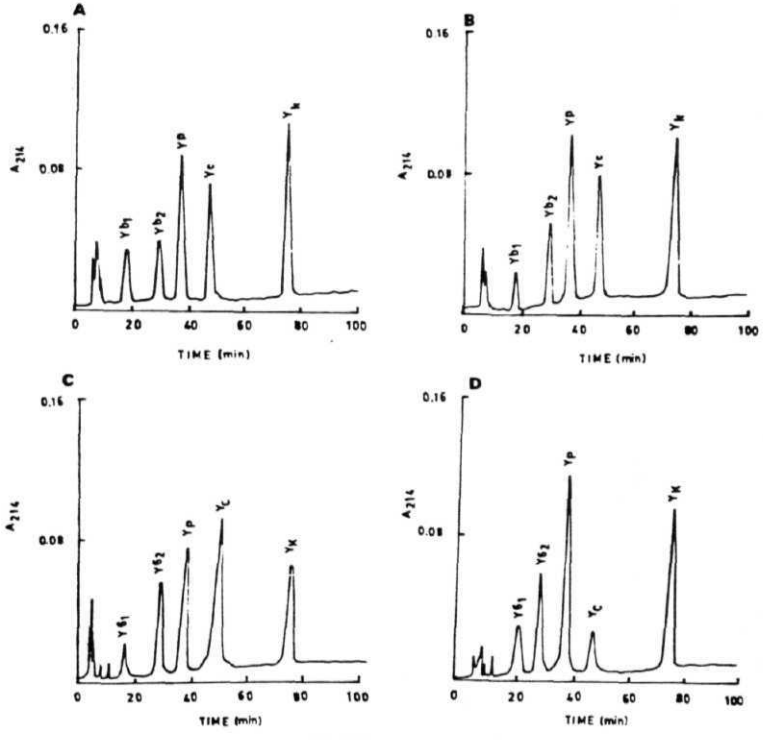
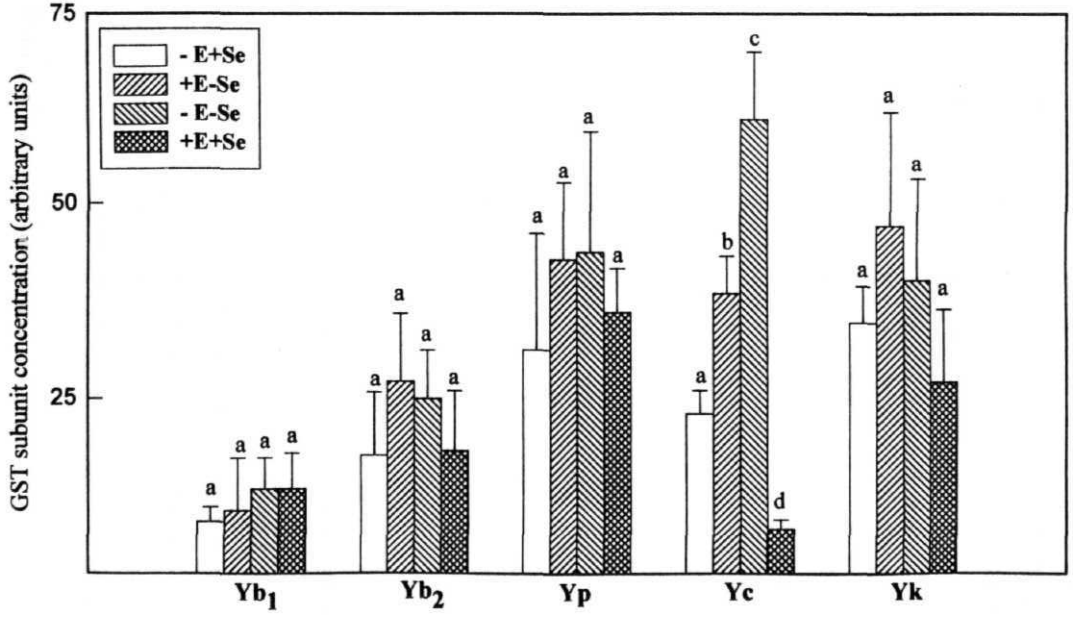


Fig. 37: Relative subunit concentration of lung affinity purified GSTs of rats fed on vit.E and/or Se supplemented and deficient diets separated on RP-HPLC



Bars represent mean ± SD of five individual experiments. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another ($p \leq 0.05$).

4.3.0.0. DISCUSSION

Vitamin E and Se deficiency is known to induce oxidative stress and simultaneously induce alternative antioxidant defenses, specifically glutathione-dependent defenses including GSTs (Mehlert and Diplock, 1985; Kim and Comb, 1993). In the present study also, induction of GSTs was observed in response to vit.E and/or Se deficiency (Chapter II, Fig. 25). However, it is not clear whether the increase in GST activity is due to an increased amount of GST protein or the activation of the enzyme systems. To unravel this, GSTs were purified by affinity chromatography and individual subunhs were separated on SDS-PAGE and RP-HPLC.

GST activity is increased in many organisms following exposure to foreign compounds. From studies of rodents, the adaptive response to chemical stress is clearly pleiotropic in character and involves the induction of many drug-metabolizing enzymes (Spencer *et al.,* 1991; Hayes *et al.,* 1993; Borroz *et al.,* 1994). Collectively, these detoxification enzymes provide protection against a diverse spectrum of harmful compounds. Evidences suggest that, besides providing protection against chemicals of foreign origin, GSTs are involved in the protection against oxidative stress. It has been reported that in Selenium- and Copper deficient rats, which are chronically exposed to increased intracellular levels of hydrogen peroxide due to lack of Se-GSH Px, marked over-expression of hepatic GST isozymes is observed (Arthur *et al.,* 1987).

Rat GST subunhs are preferentially induced by various drugs, including carcinogens like 3'-methyldiaminobenzene, 2-acetylaminofluorene (Kitahara *et al.,* 1984) and trans-stilbene oxide (Tahir *et al.,* 1989) and anticarcinogenic agents such as BHA (Kitahara *et al.,* 1984) and ethoxyquin (Kensler *et al.,* 1986).

4.3.1.0. Structural characterization of liver GSTs

In the present study oxidative stress induced by vit.E and Se deficiency resulted in the induction of hepatic GSTs, particularly Ya and Yc subunhs

containing GSTs (Fig. 28). Therefore, increased GST protein appears to be responsible for the total elevation in GST activity with the classical substrate, CDNB. Similar induction of hepatic GSTs in Se deficiency was reported by Chang *et al* (1990) and Christensen *et al* (1994). The transcriptional activation of the GST genes may account for this increase in GST subunit levels. Chang *et al* (1990) proposed that an accumulation of peroxides during Se-deficiency might possibly bind to the corresponding receptor and the receptor-ligand complex would presumably interact with chromatin to activate the corresponding genes.

The liver Ya subunit was resolved into 2 different peaks, Ya_1 and Ya_2 on reverse-phase HPLC. In the present study Ya_2 is the major form of GSTs observed in the hepatic tissue of +E+Se animals (Fig. 31D). This observation is quite contradictory to the studies reported earlier for albino rats (Hayes *et al.*, 1990), where Ya_1 was shown to be the major form of Ya GSTs. It is not clear why Ya_2 was the more abundant form in the present study. One difference between the present work and the previous reports is the use of female rats instead of male rats. These subunits seem to have different inducibility. Ya_2 is much more inducible than Ya_1 in liver tissue of vit.E and Se deficient animals (Fig. 31A,B&C).

Lai *et al* (1984) for the first time isolated a full length clone of Ya , pGTR 261, which encompases the cDNA described by Kalinyak and Taylor (1982). Further Ya subunit which was deduced from the clone cDNA pGTB 38 was described by Pickett *et al* (1984). The subunits encoded by pGTB 38 and pGTR 261 show considerable sequence homology and over their 222-amino-acid length show residue differences in only eight positions. At these positions (residues 31, 34, 96, 107, 117, 206, 207 and 219) pGTB 38 encodes glutamic acid, leucine, serine, isoleucine, arginine, proline, alanine and valine respectively, while pGTR 261 encodes aspartic acid, phenylalanine, threonine, methionine, lycine, leucine, proline and isoleucine at the respective positions. Hayes *et al* (1990) showed sequence data for Ya that include five of the eight 'difference residues' (namely 31, 34, 96, 107 and 117) and at each of these positions Ya_1 shows sequence

identity with pGTR 261. Ya_2 also includes five of the eight different residues (namely 31, 34, 107, 117 and 219), and at all of these positions Ya_2 contains the amino acids that would be predicted by pGTB 38. This provides the proof for the existence of multiple Ya-type subunits at the protein level. Hayes *et al* (1990) also reported that Ya_2 subunit was over-expressed in liver bearing aflatoxin-induced preneoplastic nodule, which was appear to be an adaptive response to aflatoxin exposure.

Waxman *et al* (1992) used gene specific oligonucleotide probes to monitor the expression of individual GST mRNA in liver and kidney of adult rats treated with different drugs. The study provided an unambiguous discrimination between the closely related GST Ya_1 and Ya_2 mRNA and have shown differential induction of these messages by different agents. They can not be distinguished at the RNA level using conventional cDNA probes (Lai *et al.,* 1984; Pickett *et al.,* 1984) or by immunoprecipitation analysis (Pemble *et al.,* 1986). GST Ya_1 is the major Ya form expressed in kidney while Ya_2 is induced in response to dexamethasone or phenobarbital treatment without any changes in Ya_1. Important differences were also observed between the responses of these two GST class a mRNA to cisplatin, which suppressed Ya_1 without any effect on Ya_2 levels. Our previous studies (Veera Reddy *et al.,* 1995) have revealed that Ya_1 subunit is induced in response to exercise training in female rats.

Regulation of GST expression is a very complex process. They are subjected to developmental control, with tissue and sex specific expression. They are responsive to physiological stress and are inducible by many drugs and chemicals. The GSTA1 (Ya_1) and GSTA2 (Ya_2) subunits in rodent liver are markedly inducible by drugs. The rat GSTA2 gene appears to contain at least four cis-acting elements in the 5'-flanking region that respond to xenobiotics. One of these elements is responsible for induction by PAH and is identical to the xenobiotic-responsive-element (XRE) found in the rat CYPIA1 gene (Rushmore *et al.,* 1990). A second element on the rat GSTA2 gene has been designated as the antioxidant-responsive element (ARE) because it mediates responsiveness to

phenolic antioxidants (Rushmore *et al.,* 1991). The third element identified in this gene is identical to the glucocorticoid-responsive element (GRE) and may render expression of GSTA2 responsive to dexamethasone (Rushmore *et al.,* 1993). A fourth element exists in rat GSTA2 which is responsible for the induction of this gene by barbiturates. The activation of transcriptional Ya gene through ARE by H_2O_2 was clearly shown by Rushmore *et al* (1990). β–Naphthoflavone, a planar aromatic compound activates the gene through either XRE or ARE, but the presence of Ah receptors and metabolism of β-naphthoflavone by cytochrome P-450 IA are required for its transcriptional activation. On the other hand, t-butyl hydroquinone, a phenolic antioxidant, activates through ARE, independently of Ah receptors or cytochrome P-450 IA1. In the presence of Ah receptors, XRE reacts with 2,3,7,8-tetrachlorodibenzo-p-dioxin, but ARE does not. These results support the proposal by Spencer *et al* (1991) that phase II drug metabolizing enzymes, including GSTs are induced by monofunctional and bifunctional inducers by different mechanisms and that induction by mono functional inducers such as t-butyl hydroxyquinone is mediated by an electrophilic signal independently of Ah receptor. The difference between ARE and XRE is its their capacity to induce phase I (cytochrome P450) gene expression. The XRE mediates induction of both phase I and phase II enzymes while ARE mediates induction of only phase II enzymes (Belinsky and Jaiswal, 1993). The induction of phase I enzymes by XRE element in response to xenobiotics may cause increased risk of mutagenecity and carcinogenecity as the products of xenobiotics activated by phase I enzymes increase the oxidative damage to DNA and membranes. The ARE-mediated phase II enzymes, on the other hand, detoxify the XRE-activated xenobiotics. Therefore, the ARE mediated increase in phase II enzymes is the safety way to stimulate the chemoprotective power of the cells.

The observed induction of Ya_2 subunit in the present study (Fig. 31A,B&C) like the induction of Ya GST in Se deficiency (Chang *et al.*, 1990), may be the livers response to rising hydroperoxide levels or free radicals when antioxidant systems are impaired due to vit.E and Se deficiency. Thus from studies

on affinity purification, western blot of crude cytosolic fractions, subunit concentration on SDS-PAGE and RP-HPLC, it is concluded that Ya_2 and Yc subunits are induced in liver tissue in response to vit.E and Se deficiency. The induction of Ya_2 subunit in vit.E and Se deficiency states might be regulated by ARE.

4.3.2.0. Structural characterization of lung GSTs

In comparison to liver, there is limited information on lung GSTs and their involvement in oxidative stress. In the present study lung GSTs resolved into four bands on SDS-PAGE, which were designated as Yc, Yb, Yk and Yp (Fig. 33). Further separation of affinity purified GSTs on RP-HPLC showed Yb_1, Yb_2, Yp, Yc and Yk among which Yp is the major subunit (Fig. 36). Both a- and u- class GSTs are found in low concentrations as reported by Cossar *et al* (1990) and Coursin *et al* (1992). Rat lung GST 7-7 is immunologically identical to the rat placental form GST-P (Robertson *et al.,* 1986).

Lung is the first and foremost tissue exposed to airborne toxicants and pollutants. Also it is more vulnerable to oxidative stress compared to other tissues. Lung is equally well equipped with antioxidant defenses, though not to the extent observed in liver. This is evident by an increase in lipid peroxides and GSSG levels in response to vit.E and Se deficiency (Fig. 18&21).

It is noteworthy that Ya which shows a high inducibility in liver is absent in lung. The lower recovery of liver vs lung GSTs (70% vs 90%) may be due to some of the isozymes specific to liver were not completely bound to affinity column or may be due to higher flow through loss (Table 8vs13). In the present study vit.E and Se deficiency significantly induced Yc subunit (7.76 fold) of a class in the lung tissue (Fig. 37). The increased GST activity might be aimed to compensate for the loss of Se-GSH Px during Se deficiency and to improve antioxidant defenses which were impaired due to vit.E deficiency.

From these studies it is concluded that GSTs exhibit tissue-specific expression. After purification, western blot analysis of cytosolic proteins, subunit concentration on SDS-PAGE and RP-HPLC it is concluded that Ya_2 and Yc subunits are induced significantly in liver and Yc subunit in lung in response to oxidative stress induced by vit.E and/or Se deficiency. The results demonstrate that Ya_1 and Ya_2 have differential inducibility, Ya_2 being more inducible than Ya_1. However it is not clear whether the GSTs induced in liver and lung tissues in response to vh.E and/or Se deficiency are involved in any antioxidant protection. Further studies on their functional characterization will throw more light on the participation of GSTs in cellular antioxidant defenses.

CHAPTER III

5.1.0.0. INTRODUCTION

While lipid peroxidation is generally associated with widespread and irreversible damage to essential cell components, enzymatically controlled lipid peroxidation is involved in the biosynthesis of eicosanoids, hormone like substances required for maintaining tissue and cellular homeostasis. The immediate oxygenation products of arachidonic acid by the way of lipoxygenase and cyclooxygenase pathways include PGG_2 and HPETE respectively. Not only do these products serve as precursors for the biosynthesis of PGs and LTs, but they also have significant influence on the activities of various enzymes associated with arachidonic acid including cyclooxygenase and lipoxygenase. Thus, an enzyme system that can reduce these reactive hydroperoxides has the potential for modulating the arachidonic acid cascade.

Glutathione peroxidases, with the potential for reducing these hydroperoxides and endoperoxides, influence the biosynthesis of biologically active eicosanoids. Se-GSH Px catalyzes the reduction of both organic and inorganic hydroperoxides. In addition to these a group of Se-independent GSH Pxs (non-Se-GSH Px) associated with certain forms of GSTs are involved in the reduction organic hydroperoxides (Lawrence and Burke, 1976). Both types of peroxidases are ubiquitously distributed in animals. The cellular Se-GSH Px is located primarily in the cytosolic and mitochondrial compartments, while the non-Se-GSH Px has been identified in both cytosolic and microsomal cell fractions (Reddy *et ah,* 1981). The relative abundance of these two types of GSH Pxs within a given tissue is species specific. The relative contribution of these two enzymes in antioxidant defenses can best be studied by knocking out these enzymes one at a time. Induction of Se deficiency forms one such situation wherein Se-GSH Px activity is eliminated. As shown in the second chapter, Se deficiency and thus elimination of Se-GSH Px resulted in the induction of GSTs. The GSTs have been implicated in the synthesis of prostaglandins including $PGF_{2\alpha}$. $PGF_{2\alpha}$ is a biological mediator in a variety of physiological functions including contractions of

uterine smooth muscle and pulmonary arteries. Research from Reddy's group has provided evidence that the GSH Px activity of GSTs is associated with the reduction of PGH2 to $PGF_{2\alpha}$, with the α-class or Ya-containing subunits being the most active (Burgess *et al.*, 1987; Chang *et al,* 1987a; Hong *et al,* 1989). In addition to their role in PG biosynthesis, GSTs also play a role in lipoxygenase pathway at the level of reduction of hydroperoxides and in the formation of peptidoleukotrienes. GSTs, especially Yb containing subunits were shown to conjugate GSH to LTA_4 to produce LTC_4, the most potent vasoconstricting and branchoconstricting compound (Chang *et al,* 1987b). Hence in the present study GSTs from the various dietary oxidant stress conditions were screened for their potential impact on the enzymatic lipid peroxidation pathways of the arachidonic acid.

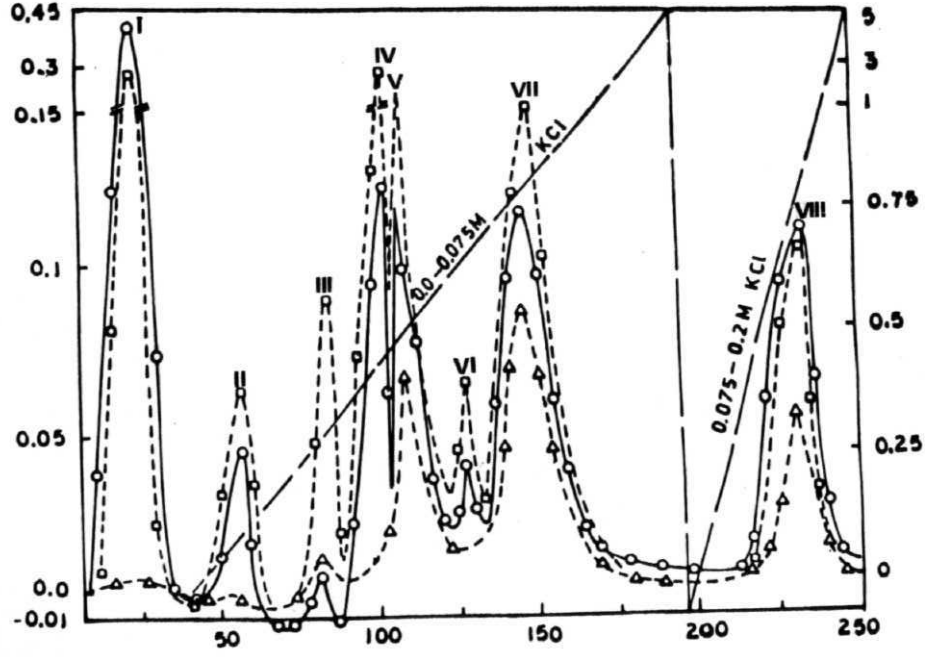
5.2.0.0. RESULTS

5.2.1.0. CM-Cellulose column chromatography

The affinity purified GSTs from liver cytosol of +E+Se rats were further separated into different isozymes on CM-cellulose column chromatography. As shown in the typical chromatogram (Fig. 38) eight GST activity peaks, designated as peaks I to VIII according to the ordeT of elution, were consistently resolved on the CM-cellulose column chromatography with two linear salt gradients used in succession, i.e., 0 to 75 mM KC1 and 75 to 200 mM KCl, in 10 mM NaH_2PO4, pH 6.0. Peak I isozyme, which did not bind to the CM-cellulose column is referred to as the anionic GST isozyme. The other isozyme peaks which were eluted by the salt gradient are referred to as the cationic GST isozymes.

SDS-PAGE analysis of each peak fraction demonstrated that all of them were electrophoretically pure (Fig. 39). Peak I, II, IV and VI were composed of Yb subunits only. Peak HI was a Ya homodimer. Peak VII and VIII were Yc homodimers. Peak V consisted of equal amounts of Ya and Yc subunits as previously reported for transferases (Bass *et al,* 1977; Tu and Reddy, 1985).

Fig. 38: Separation of affinity purified hepatic GST isozymes of +E+Se rats on CM- cellulose column chromatography

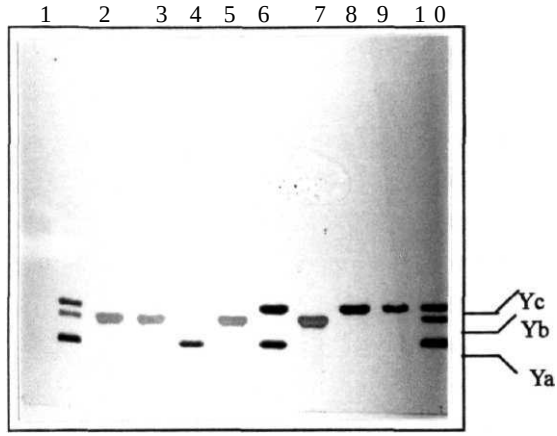


A_{280} (0–0)

FRACTION NUMBER

Activity with CDNB µmoles/min/mL (□–□)
Activity with CHP µmoles/min/mL (Δ–Δ)

Fig.39 : SDS-PAGE of liver GST isozymes +E+Se rats separated on CM-cellulose column chromatography



Lane: 1. Rat liver affinity purified GSTs
Peak fraction #

2. 21	3.58	4.85
5. 99	6. 107	7. 128
8. 143	9.227	

10. Rat liver affinity purified GSTs

5.2.2.0. DE 52 cellulose column chromatography

The anionic form of GSTs obtained from the CM-cellulose column flow through fractions were subjected to anion-exchange chromatography on DE 52 cellulose column for further separation. No protein was observed in the effluent prior to the start of a linear gradient of 0-100 mM KC1 in 10 mM Tris-HCl, pH 8.0. All the protein bound to the column was eluted at approximately 60 mM KC1 as a single peak, coincident with the GST activity (Fig. 40).

5.2.3.0. GST catalyzed reduction of classical substrates

The substrate specificity studies have shown that , the isozymes containing Ya and/or Yc subunits can be clearly distinguished from the isozymes containing Yb subunits by their relatively low activities with DCNB and high non-Se-GSH Px activity (Table 15&16). These results indicate that all of the rat liver GST isozymes may be classified into two groups, Ya and Yc containing isozymes and Yb subunit containing isozymes according to their physical and chemical properties as well as biological activities. The YaYc heterodimeric protein is always eluted after the Ya homodimer and before Yc homodimer during CM-cellulose chromatography (Fig. 38). The activity levels with different substrates for peak V (YaYa) are approximately intermediate between those of peak III (YaYc) and peak VII (YcYc). Peak III (Ya homodimer) is characterized by having the highest activity with l,2-dichloro-4-nitrobenzene; 1,2-epoxy-3-(p-nitrophenoxy)propane; Δ^5-androstene-3,17-dione; bromosulfopthalein, p-nitrophenyl acetate and 13-hydroperoxyoctadecadienoic acid (13-HPODE) as well as $PGF_{2\alpha}$ formation (Table 15&16). Peak VII (Yc homodimer), on the other hand, showed the highest activity with ethacrynic acid as well as organic hydroperoxides (Table 15&16). In contrast to the Ya and/or Yc containing isozymes, peaks I, II, IV and VI all contained only Yb subunits. However, they eluted at different salt concentrations on CM-cellulose columns indicating their varied structural characteristics. For example, peak I was eluted in the CM flow through fractions, whereas peak II, IV & VI were eluted at different salt concentrations. In addition to their structural

Fig. 40: Anion exchange chromatography of +E+Se rat liver CM-cellulose column flow through fraction on DE 52 cellulose column chromatography

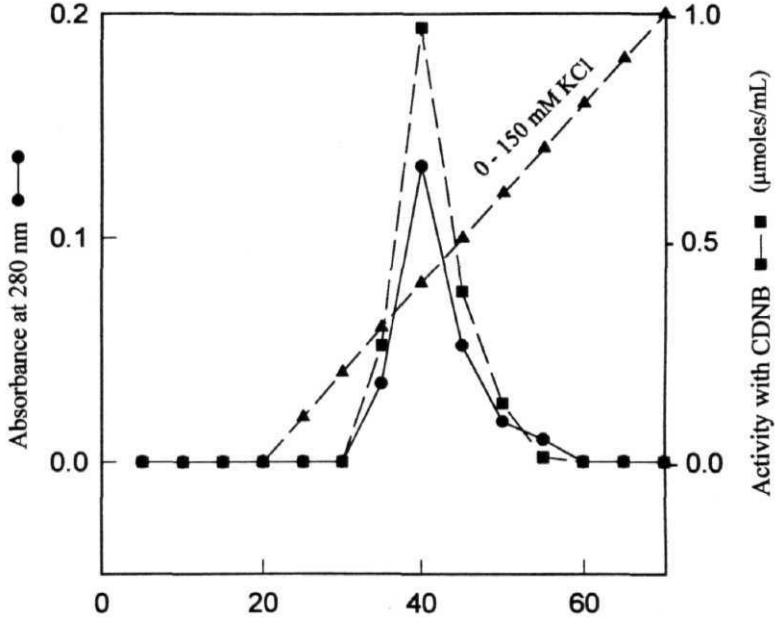


TABLE 15 : SUBSTRATE SPECIFICITIES OF +E+Se RAT LIVER GST ISOZYMES SEPARATED ON CM-CELLULOSE COLUMN CHROMATOGRAPHY TO CLASSICAL SUBSTRATES

Substrate	I YbYb	II YbYb	III YaYa	IV YbYb	V YaYc	VI YbYb	VII YcYc	VIII YcYc
1,2-Dichloro-4-nitrobenzene	0.17	1.10	0.82	1.07	0.6	1.96	0.06	0.07
1-Chloro-2,4-dinitrobenzene	3.66	14.44	21.0	17.1	19	19.6	8.88	2.17
Δ^5-Androstene-3,17-dione	0.01	0.02	1.0	0.03	0.8	0.05	0.04	0.03
1,2-Epoxy-3-(p-nitrophenoxy)propane	0.30	ND	0.10	0.095	0.1	0.07	ND	ND
4-Nitropyridine-N-oxide	0.015	0.12	0.03	0.12	0.1	0.14	0.14	0.02
p-Nitrobenzyl chloride	0.78	3.27	1.86	3.84	1.3	0.83	1.20	1.01
Ethacrynic acid	0.15	0.36	0.70	0.44	0.9	0.13	1.25	0.9
p-Nitrophenyl acetate	0.20	0.2	0.38	0.42	0.3	0.57	0.20	0.17
Bromosulfophthalein	0.01	0.02	0.06	0.02	0.03	ND	ND	0.01

Values expressed as μmoles/min/mg protein.

TABLE 16: SUBSTRATE SPECIFICITIES OF +E+Se RAT LIVER GST ISOZYMES SEPARATED ON CM-CELLULOSE COLUMN CHROMATOGRAPHY TO CONVENTIONAL SUBSTRATES

Substrate	I YbYb	II YbYb	III YaYa	IV YbYb	V YaYc	VI YbYb	VII YcYc	VIII YcYc
Cumene hydroperoxide	0.56	0.040	5.12	1.05	5.43	1.42	8.0	10.5
t-Butyl hydroperoxide	0.05	0.21	1.32	0.41	1.4	0.71	2.25	1.90
13-HPODE	0.04	0.03	2.1	0.11	1.30	0.18	0.61	0.3
* PGH_2 to $PGF_{2\alpha}$	15	125	1520	250	552	120	328	287
* LTA_4 Me	123	7.9	6.2	69.7	1.6	ND	ND	ND

Values expressed as μmoles/min/mg protein.
* Values expressed as nmoles/min/mg protein.

differences, they also showed varied functional characteristics. For example, peak I showed highest activity towards 1,2-epoxy-3-(p-nitrophenoxy)propane and LTA_4 Me; followed by peak IV with less activity and peak VI with no activity (Table 16). Similarly peak I, IV and VI showed relatively low, intermediate and very high activities respectively with DCNB, 4-nitropyridine n-oxide, Δ^5-androstene-3,17-dione and p-nitrophenyl acetate (Table 15).

5.2.3.1. GST catalyzed reduction of cumene hydroperoxide

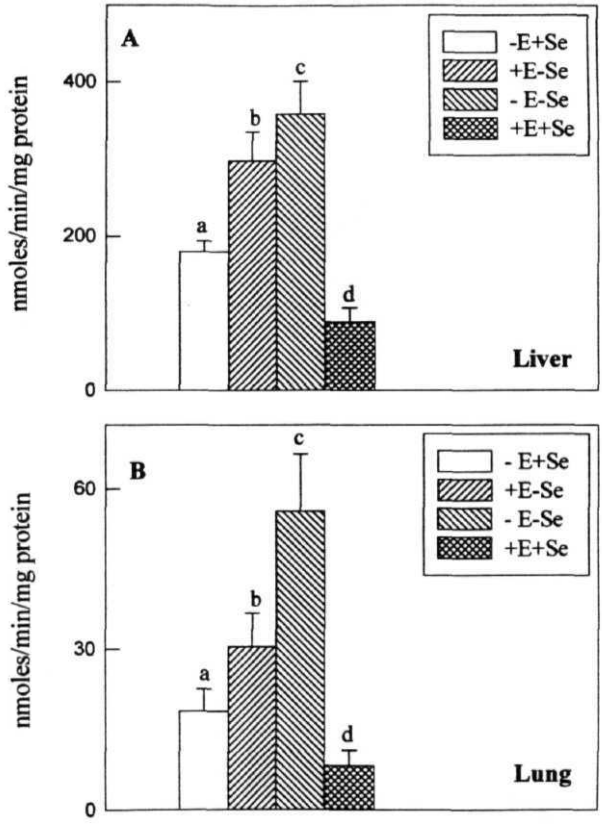
Se-GSH Px acts both on H_2O_2 and organic hydroperoxides whereas α-class GSTs act only on organic hydroperoxides which was designated as non-Se-GSH Px. The non-Se-GSH Px activity when measured with cumene hydroperoxide in the crude cytosolic fractions showed very high activity in the liver tissues of -E+Se (103 %), +E-Se (236 %) and -E-Se (304 %) animals when compared to the +E+Se animals (Fig. 41 A). Similarly, in lung tissue non-Se-GSH Px activity was induced in -E+Se (121 %), +E-Se (267 %) and -E-Se (572 %) animals (Fig. 41B). The activity levels in -E-Se were much higher when compared to that of +E-Se animals.

In the affinity purified GSTs, a clear induction of peroxidase activity is observed in liver and lung tissues of vit.E and/or Se deficient animals (Fig. 42A&B), however, the induction being much higher in the cytosolic fractions.

5.2.3.2. GST catalyzed reduction of 13-hydroperoxyoctadecadienoic acid (13-HPODE)

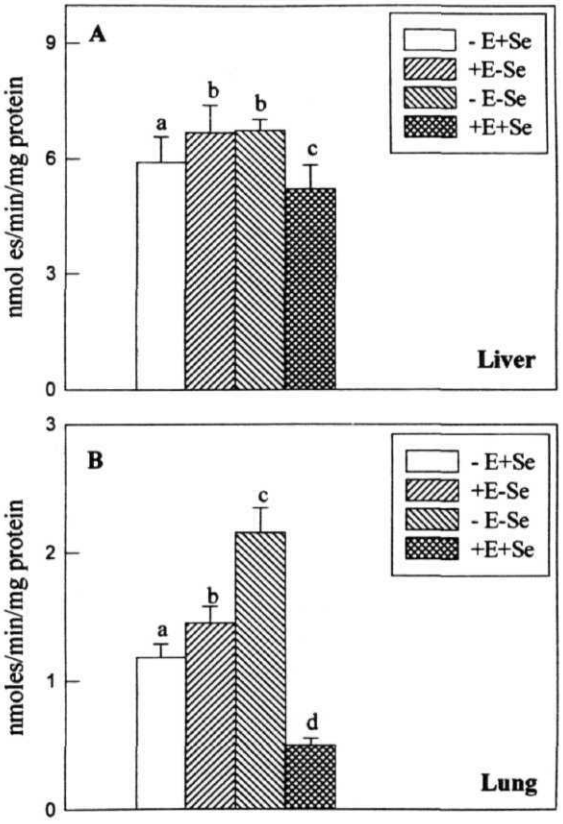
The total GSH Px activity measured with 13-HPODE in the crude cytosolic fractions, decreased in the liver tissues of +E-Se (- 64 %) and -E-Se (-61 %) animals when compared to that of +E+Se animals (Fig. 43A). Similarly in lung tissue, significantly lower levels of peroxidase activity were observed in +E-Se (-51%) and -E-Se (-30 %) groups compared to that of +E+Se animals (Fig. 43B).

Fig. 41: Activity levels of GSTs with CHP in cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



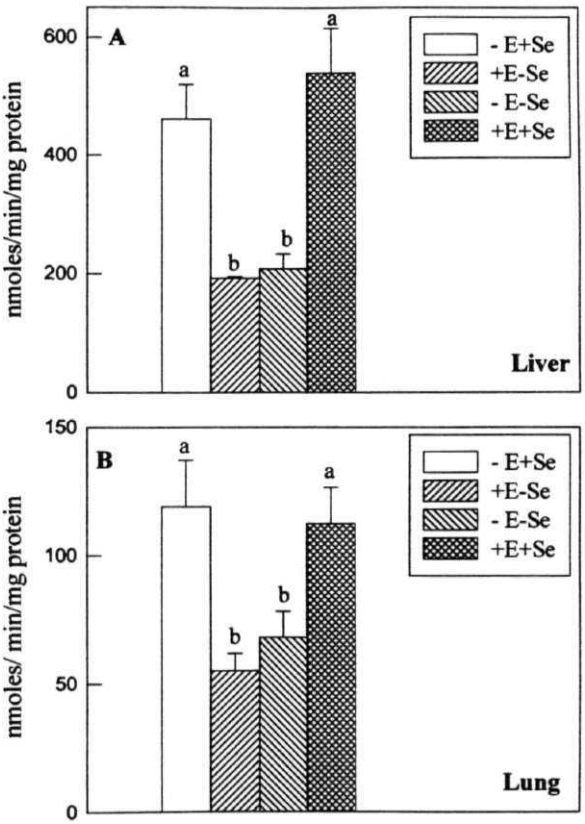
Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 42: Activity levels of non-Se-GSH Px with CHP in the affinity purified GSTs of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



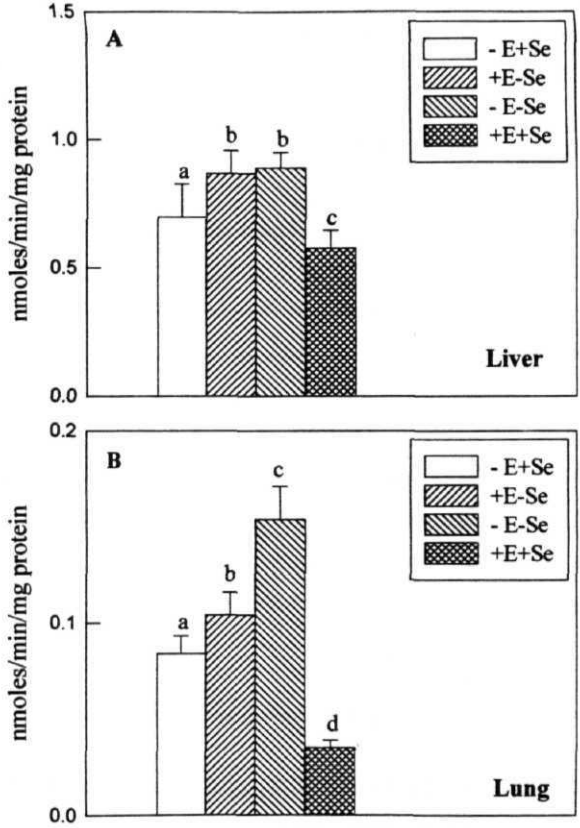
Each value is the mean + SD of six individual observations.
Data was analyzed with a one way ANOVA followed by SNK test.
Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 43: Activity levels of GSTs with 13-HPODE in cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 44: Activity levels of GSH Px with 13-HPODE in the affinity purified GSTs of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

In contrast, the affinity purified GSTs showed a clear induction of peroxidase activity in liver tissues of -E+Se (21 %), +E-Se (50%) and -E-Se (54 %) and lung tissues of -E+Se (140 %), +E-Se (197 %) and -E-Se (340 %) when compared to the corresponding tissues from +E+Se animals (Fig. 44A&B).

5.2.3.3 GST catalyzed reduction of 15-hydroperoxyeicosatetraenoic acid (15-HPETE)

Total GSH Px activity levels in liver tissues were significantly lower in +E-Se (-61 %) and -E-SE (-56 %) animals when compared to that of +E+Se animals (Fig. 45A). Similarly, significantly lower levels of total GSH Px were observed in lung tissues of +E-Se (-56 %) and -E-Se (-27 %) animals when compared to that of +E+Se animals (Fig. 45B). GSH Px activity towards fatty acid hydroperoxides decreased significantly in liver and lung crude cytosols Se deficient animals , but not in those of vit.E deficient animals.

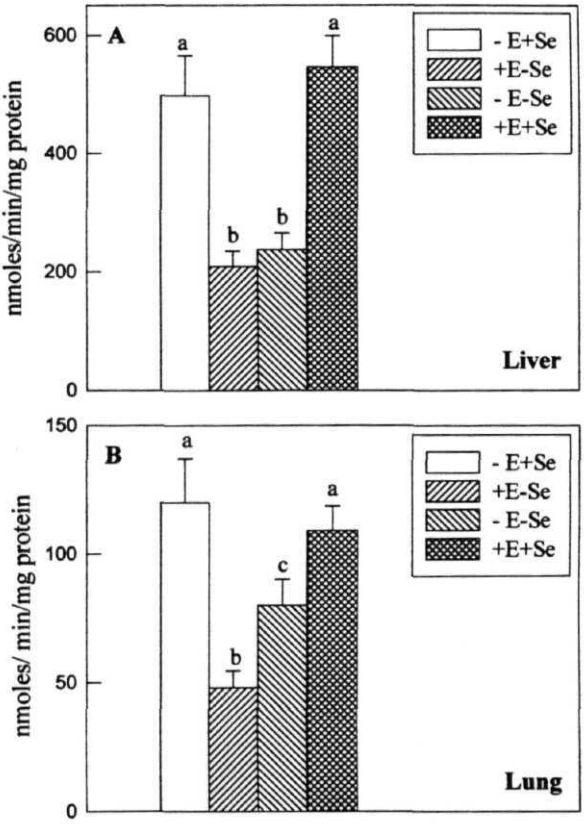
In contrast, the affinity purified GSTs showed significantly increased peroxidase activity in liver tissues of all vit.E and Se deficient [-E+Se (21 %), +E-Se (32 %) and -E-Se (34 %)] animals when compared to +E+Se animals (Fig. 46A). Similarly higher peroxidase activity levels were observed in lung tissues of -E+Se (91 %), +E-Se (136 %) and -E-Se (249 %) animals compared to that of +E+Se animals (Fig. 46B).

5.2.3.4. GST catalyzed prostaglandin formation

Equal amounts of affinity purified liver and lung GSTs from vit.E and/or Se supplemented and deficient animals were analyzed for GST catalyzed prostaglandin formation. The prostaglandins were identified by the Rf values corresponding to the prostaglandin standards separated on TLC (Fig. 47).

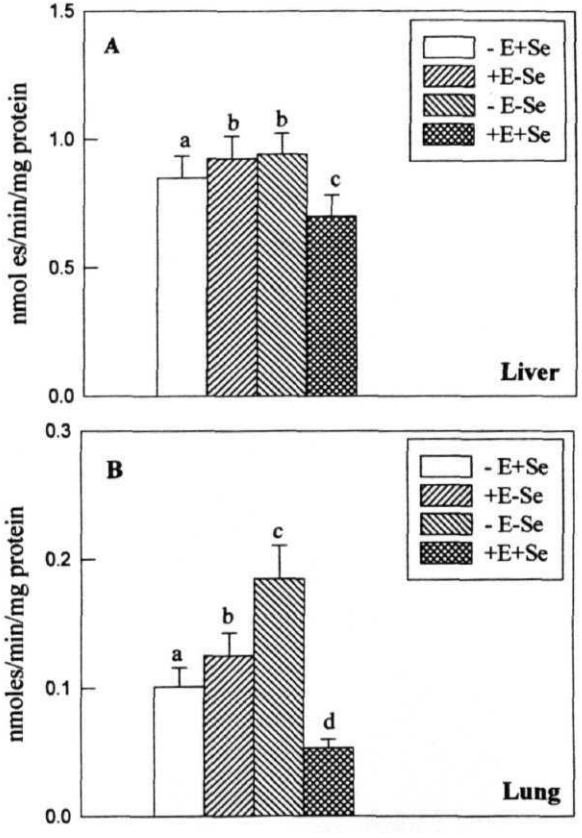
The GST catalyzed $PGF_{2\alpha}$ forming activity in the affinity purified GSTs were significantly higher from liver tissues of -E+Se (32 %), +E-Se (51 %) and -E-Se (92 %) animals compared to that of +E+Se animals (Table 17). This correlates well with the observation that the Ya subunit, which exhibits maximum

Fig. 45: Activity levels of GSTs with 15-HPETE in cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 46: Activity levels of GSH Px with 15-HPETE in the affinity purified GSTs of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets



Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test Bars labelled with same letter are not significantly different from one another ($p < 0.05$).

Fig. 47: Separation of prostaglandin standards on TLC

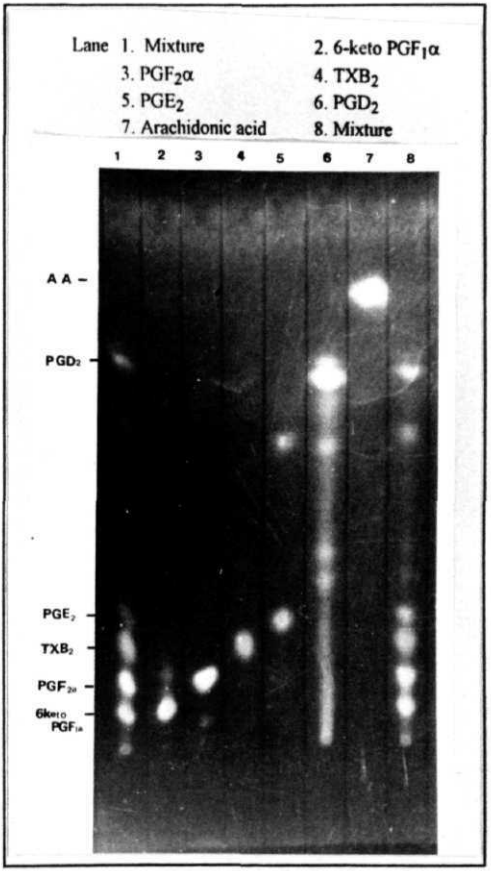


Table 17: Synthesis of prostaglandins catalyzed by liver affinity purified GSTs of vit.E and/or Se supplemented and deficient rats

PG	-E+Se	+E-Se	-E-Se	+E+Se
PGD_2	34.7 ± 6.93[a]	32.06 ± 7.57[a]	33.12 ± 5.46[a]	39.48 + 9.1[a]
PGE_2	42.5 ± 6.43[a]	43.06 ± 6.39[a]	46.47 + 6.61*	41.66 ± 7.2[a]
$PGF_{2\alpha}$	22.8 ± 2.72[a]	24.84 ±4.14*	30.40 + 4.1[b]	15.80 ± 3.75[c]

Values represent mean ± SD of five individual experiments and are expressed in terms of percent ratios of total PGs formed. Data was analyzed with a one-way ANOVA followed by SNK test. Significance was set at $p \leq 0.05$. The same alphabet denotes no significant difference between the two groups in each row.

Table 18: Synthesis of prostaglandins catalyzed by lung affinity purified GSTs of vit.E and/or Se supplemented and deficient rats

PG	-E+Se	+E-Se	-E-Se	+E+Se
PGD_2	88.62 ±15.8*	88.11 ± 15.5[a]	89.24 ± 19.11[a]	88.74 ± 22.4[a]
PGE_2	8.52 + 1.60[a]	8.88 ± 1.75[a]	8.34 ± 2.4*	8.37 ±0.72*
$PGF_{2\alpha}$	2.84 ± 0.69[a]	2.99 + 0.75[a]	2.56 ± 0.23[a]	2.89 ± 0.24[a]

Values represent mean ± SD of three individual experiments and are expressed in terms of percent ratios of total PGs formed. Data was analyzed with a one-way ANOVA followed by SNK test. Significance was set at $p \leq 0.05$. The same alphabet denotes no significant difference between the two groups in each row.

peroxidase activity, was also induced significantly in the livers from vit.E and Se deficient animals (II chapter).

$PGF_{2\alpha}$ forming activity was also investigated with rat lung affinity purified GSTs from vit.E and Se deficient and supplemented animals. In contrast to the results observed in liver, $PGF_{2\alpha}$ synthesis was barely detectable in the lung tissue (Table 18). No significant changes were observed in PGE2 or PGD2 formation in both lung and liver tissues in response to vit.E and/or Se deficiency (Table 17&18).

5.2.3.5. GST catalyzed leukotriene formation

Among various rat liver isozymes, the anionic isozyme (peak I), a homodimer of Yb subunit, showed the highest LTC4 Synthase activity, followed by peak IV GST isozymes (Table 16). When 5,6 LTA_4 methyl ester was incubated with equal amounts (100 µg) of liver affinity purified GSTs from vh.E and/or Se deficient animals for 10 min in the presence of GSH, most of the LTA4 methyl ester was converted into an acid-resistant compound, LTC_4 Me, which eluted around 5 min on RP-HPLC (Fig. 48). The UV-absorption spectrum of the compound showed a typical LTC4 spectrum with maximum absorption at 280 nm and shoulders at 270 and 292 nm. Basing on the UV/VIS spectrum and co.chromatography with authentic standard, the compound with RT 5 min was identified as LTC_4 Me. In contrast to liver, the lung GSTs showed significantly higher LTC_4 Synthase activity. However, there were no significant differences in LTC_4 Synthase activity of GSTs in vit.E and/or Se deficient and supplemented animal tissues (Table 19).

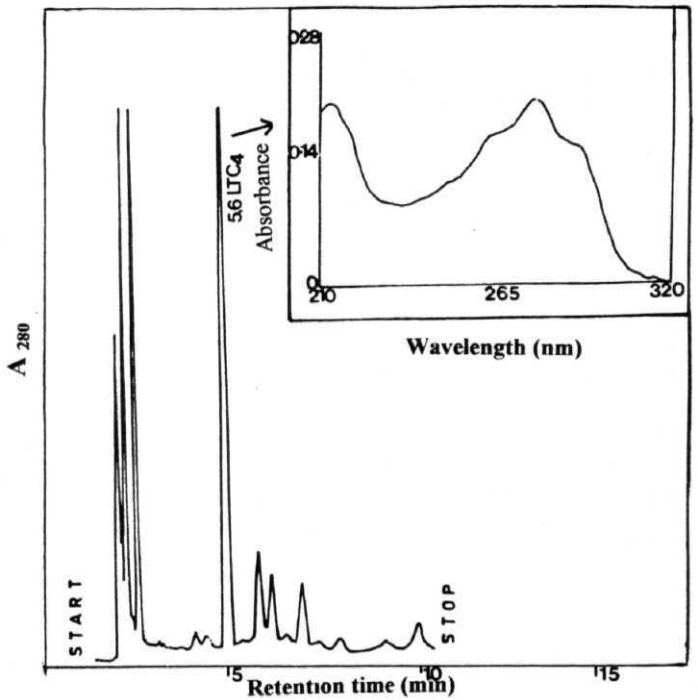
53.0.0. DISCUSSION

5.3.1.0. Functional characterization of liver GSTs

The non-Se-GSH Px activity was significantly increased in liver cytosol fractions and affinity purified GSTs of vit.E and Se deficient animals (Fig.

Fig. 48: RP-HPLC analysis of GSTs catalyzed LTC_4 formation

Inset: UV/VIS absorption spectrum of 5,6 LTC_4 peak in methanol



Column: ODS, μ Bondapak (Waters)
Solvents: Methanol Water:Acetic acid (65:35:0.1) pH 5 7
Flow rate: 280 nm

Table 19: LTC_4 Synthase activity of affinity purified liver and lung GSTs of vit.E and/or Se supplemented and deficient rats

TISSUE	-E+Se	+E-Se	-E-Se	+E+Se
Liver	4.40 ± 0.8[1]	4.6 ± 1.9[a]	4.64 + 1.54[a]	5.6 + 1.6[a]
Lung	12.8 + 0.4[a]	11.6 + 3.4[a]	15.4 + 3.6[a]	12.6 ± 3.6"

Values are mean + SD of five individual observations and are expressed as nmoles/min/mg protein. Data was analyzed with a one-way ANOVA followed by SNK test. Significance was set at $p < 0.05$. The same alphabet denotes no significant difference between the two groups in each row.

41&42). This increased non-Se-GSH Px activity correlated well with increase in the Ya and Yc subunit containing GSTs (chapter II). Also Ya and Yc subunits exhibit maximum activity towards CHP as substrate (Table 16).

In addition to Se-GSH Px, non-Se-GSH Px activity associated with GSTs also reduce ferry acid hydroperoxides. Fatty acid hydroperoxidase activity measured in the crude cytosol of vit.E and/or Se deficient and supplemented animal tissues with 13-HPODE and 15-HPETE showed significantly reduced activity levels in Se deficient animals (+E+Se; -E-Se) compared to Se supplemented animals (-E+Se; +E+Se) (Fig. 43&45). The fetty acid hydroperoxidase activity measured in the crude cytosol of Se supplemented animals (-E+Se; +E+Se) is contributed by both Se-GSH Px as well as non-Se-GSH Px activity of GSTs. Thus a more accurate measure of GSH Px activity would be achieved by subtracting H_2O_2 (Se-GSH Px) activity as is traditionally done in estimating non-Se-GSH Px activity with CHP. Indeed, such a calculation would closely approximate the value measured when the fetty acid hydroperoxidase activities were measured in the affinity purified fraction. Fatty acid hydroperoxidase activity of GSTs was increased by 54% with 13-HPODE and 34% with 15-HPETE in vit.E and /or Se deficient animals compared to supplemented groups. This induction of fetty acid hydroperoxidase activity in vit.E and/or Se deficient animals must have been contributed by Ya and Yc subunits in liver tissue as these isozymes exhibit maximum peroxidase activity (Table 15) and these are the subunits induced in liver tissues of vit.E and/or Se deficient animals (chapter II). It is to be noted that all fetty acid hydroperoxides are good substrates for Se-GSH Px, and is 50 times more efficient than non-Se-GSH Px in catalyzing the reduction of fetty acid hydroperoxides.

In addition to the fetty acid hydroperoxidase activity, GSTs also were reported to be involved in the biosynthesis of prostaglandins and leukotrienes. Ya subunits of GSTs were shown to be involved in PGE_2 and $PGF_{2\alpha}$ synthase activity (Chang *et al.*, 1987a). In the present study liver affinity purified GSTs of +E+Se

animals when incubated with PGH_2 resulted in the formation of PGE_2, PGD_2 and $PGF_{2\alpha}$, the relative abundance being in the same order (Table 17). In the vit.E and/or Se deficient animals, there was significantly higher formation of $PGF_{2\alpha}$ compared to that of supplemented animals with no change in the formation of PGE_2 and PGD_2 (Table 17). Hope *et al* (1975) have reported increase in the formation of PGE_2 and $PGF_{2\alpha}$ in rat blood in response to vit.E deficiency. Cooper and Carpenter (1987) found no effect of diet on PGE_2 production by sertoli cells isolated from the testes of rats maintained on vit.E altered diets. Hepatic $PGF_{2\alpha}$ was significantly increased along with GST activities in phenobarbital treated rats which suggests that induction of Ya containing GSTs may play a physiologically important role in the synthesis of $PGF_{2\alpha}$, which may account, at least in part, for the tumor-promoting effects of phenobarbital (Hendrich *et al.,* 1991).

No significant changes were observed in the leukotriene synthesis of vit.E and/or Se deficient animals compared to that of +E+Se animals (Table 19). The formation of LTC4 has been demonstrated in mouse myocytoma cells, rat basophilic leukemia cells, rat mononuclear cells and in human and guinea pig lungs (Piper, 1984; Dahinden *et al.,* 1985). GST Yn_1-Yn_1 purified from rat brain has the highest LTC4 synthase activity so far found in rat cytosolic GSTs and accounts for the majority of LTC_4 synthase activity in the brain (Tsuchida *et al.,* 1987). In the present study peak I with Yb_2Yb_2 subunits (CM-cellulose fractions) showed maximum LTC4 Synthase activity in the liver tissue (Table 15). As shown in the chapter II, liver contains very little or no Yn_1Yn_1 isozymes and Yb_2Yb_2 is not induced in vit.E and/or Se deficiency. Thus GSTs induced by antioxidant deficiency are unlikely to alter LT synthesis directly.

The accumulated peroxides in Se deficiency could activate the transcription of the specific GST genes which would result in non-Se-GSH Px activity. This further implies that environmental factors and pathological conditions which result in increased lipid peroxidation or otherwise increased peroxide levels might also induce changes in GST expression, especially those

involved in their reduction and detoxification. In the present study induction of Ya and Yc subunits in the liver tissue of vit.E and/or Se deficient animals appears to be aimed at the reduction of hydroperoxides as well as endoperoxides. GSTs with Ya and Yc, more so with Yc, are more involved in the reduction of hydroperoxides while GSTs with Ya subunits being more specific for reduction of endoperoxides.

5.3.2.0 Functional characterization of lung GSTs

The lung tissue of vit.E and/or Se-deficient animals is under increased oxidative stress as evidenced by increased GSSG levels and reduced Se-GSH Px activity levels (I chapter, Fig. 21&19). The increased non-Se-GSH Px activity of GSTs with CHP as the substrate in vit.E and/or Se deficient animals observed in the present study (Fig. 41B&42B) may be a compensatory mechanism to protect the lung tissue from the increased oxidative stress. The induction of GSTs with Yc subunits in lung tissue of vit.E and/or Se-deficient animals (II chapter) supports such a possibility as they exhibit very high peroxidase activity. Fatty acid hydroperoxidase activity was measured with affinity purified lung GSTs with 13-HPODE as well as 15-HPETE (Fig. 44&46). Fatty acid hydroperoxidase activity was much higher in Se-deficient animal tissue compared to Se-supplemented ones. This pattern is very much similar to that observed for liver tissue except that it correlated with elevated Yc rather than predominantly Ya GSTs.

GSTs can reduce electrophilic substrates like reactive alkenes; a,P unsaturated aldehydes, including highly toxic 4-hydroxyalkenals and epoxides formed during lipid peroxidation. Among all the GST isozymes, GST 8-8 (YkYk) showed maximum activity towards 4-hydroxynonenal, which is a product of peroxidative degradation of arachidonic acid (Stenberg *et al.*, 1992). Rat lung which contains YkYk isozyme, is thus involved in the reduction of hydroxyalkenals produced in lipid peroxidation. Even though Yk was not elevated in vit.E and/or

Se deficiency, the increased activity during oxidative stress could partially account for elevated GSSG in lung (chapter I).

Earlier studies have shown that glutathione peroxidase activity of GSTs was restricted to ferry acid hydroperoxides, which suggests that membrane phospholipids must be first cleaved by phospholipase A_2 before they could be utilized as substrates by GSTs (Tan *et al.,* 1984). However the later studies have shown that the α-class GSTs including those of human lung can effectively reduce the intact phospholipid hydroperoxides through GSH Px activity (Singhal *et al.,* 1992). Hence it can be suggested that the α-class GST isozymes (Yc & Yk) play a predominant role in detoxification of lipid peroxides particularly in lung tissue. GST *n* which constitutes about 90% GST activity towards electrophilic substrates, on the other hand is known for the detoxification of xenobiotics (Batist *et al,* 1986; Tsuchida and Sato, 1992; Zhang, 1994).

In rat lung, however, dietary vit.E and/or Se status had no effect on the conversion of PGH_2 to $PGF_{2\alpha}$ or in the formation of other prostaglandins (Table 18). This result can be explained by the observation that rat lung lacks Ya subunit (Reddy *et al.,* 1982), which is known for endoperoxidase activity. This association of prostaglandin endoperoxide reduction with Ya subunit is consistent with the feet that rat brain cytosolic GSTs which lack this subunit also showed very little $PGF_{2\alpha}$ forming activity (Thyagaraju *et al,* 1986b).

Compared to liver tissue, lung tissue of +E+Se animals showed 3-fold higher LTC_4 synthase activity (Table 19). This may be due to the presence of GSTs with more of Yb subunits than in the liver tissue, which exhibit very high LTC4 synthase activity. LTC« synthase activity in vit.E and/or Se deficient animals showed no significant differences when compared with +E+Se animals. This observation coincides well with the data on GST Yb subunits, where no significant changes were observed (chapter II).

From these studies it can be concluded that, induction of Ya_2 and Yc subunits in liver and Yc subunits in lung tissue with increase in peroxidase activity in vit.E and/or Se deficient animals compared to vit.E and Se supplemented animals, is a compensatory mechanism towards impaired antioxidant defenses as a result of nutritional deficiency.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Glutathione S-transferases (GSTs, EC 2.5.1.18) constitute a complex family of enzymes with multiple functions that are mostly associated with the biotransformation/detoxification of a wide spectrum of xenobiotics. They exist in complicated but distinct isozyme forms in different mammalian tissues. They are distinguishable by catalytic and immunological properties and by primary structure as well. Though their role in detoxification of xenobiotics is well known, their involvement in antioxidant defenses especially during oxidative stress is not well defined. In the present study structural and functional characterization was undertaken for GSTs from liver and lung tissues of animals subjected to oxidative stress. Female albino rats at weanling stage were fed on diets deficient and/or supplemented with vit.E and/or Se for a period of 13 weeks to induce oxidative stress by impairing vit.E and Se-GSH Px antioxidant activities.

1. Feeding of female albino rats with vit.E and/or Se deficient diets for a period of 13 weeks reduced vit.E and Se levels in hepatic and lung tissues to almost negligible levels.

2. Vitamin E and Se deficiency resulted in the stimulation of enzymes involved in GSH metabolism.

3. Vitamin E and/or Se deficiency in female albino rats resulted in oxidative stress in liver and lung tissues as evidenced by increased levels of lipid peroxides and decreased Se-GSH Px levels. Of these two, lung tissue is relatively more susceptible to oxidative damage.

4. Oxidative stress by Se deficiency resulted in the induction of GSTs both in liver and lung tissues as evidenced by increased specific activity of GSTs in cytosolic fractions, increased total affinity purified GST protein and decreased purification fold of Se deficient groups.

5. Western blot analysis of crude cytosol fractions, SDS-PAGE and RP-HPLC analysis of affinity purified proteins of vit.E and/or Se deficient animals revealed induction of Ya_2 and Yc subunits in liver tissue compared to that of +E+Se animals.

6. In the lung tissue, which lacked Ya subunits, only GSTs with Yc subunits were induced in response to vit.E and/or Se deficiency.

7. Substrate specificity studies have revealed that GSTs with Ya subunits exhibit maximum activity with fatty acid endoperoxides such as PGH2, whereas Yc containing subunits showed maximum activity with organic hydroperoxides.

8. Substrate specificity of cytosol fractions and affinity purified GSTs from liver and lung tissues of vit.E and Se deficient animals showed increased activity with CHP, the organic hydroperoxides including 15-HPETE and 13-HPODE..

9. The increase of GSTs with peroxidase activity of GSTs towards organic peroxides like CHP, 15-HPETE and 13-HPODE observed in vit.E and/or Se deficient animals is consistent with an adaptive mechanism to enhance antioxidant defenses under oxidative stress.

10. Liver GSTs from vit.E and/or Se deficient animals had significantly higher $PGF_{2\alpha}$ synthase activity in comparison to vit.E and Se supplemented animals. This observation coincides well with the increased GST Ya subunit in vit.E and/or Se deficient animals, which exhibits endoperoxidase activity. No significant differences, however, were observed in PG formation in the lung tissue of vit.E and/or Se deficient animals.

11. LTC_4 synthase activity of GSTs when measured in liver and lung tissues showed higher activity in the lung compared to liver tissue. This was attributed to high concentration of Yb subunits present in lung tissue, which exhibits higher LTC_4 synthase activity.

From these studies it is concluded that, GSTs in addition to their role in detoxification of xenobiotics, play a predominant role in antioxidant defense and are selectively induced during oxidative stress.

REFERENCES

Alfthan G. (1984) *Anal. Chim. Acta* 165, 187-194.

Arriess G.R., Chagchitt C, Chen L.-C. and Chow C.K. (1988) *Nutr. Res.* 8, 653-661.

Arthur J.R., Morrice P.C., Nocol F., Beddows S.E., Boyd R., Hayes J.D. and Beckett G.J. (1987) *Biochem. J.* 248, 539-544.

Arun S. and Ajay K. (1990) *Indian J. Exp. Biol.* 28, 780-782.

Awad J.A., Morrow J.D., Hill K.E., Roberts L.J. and Burk R.F. (1994) *J. Nutr.* 124, 810-816.

Awasthi Y.C. and Singh S.V. (1985) *Comp. Biochem. Physiol.* 82B, 17-23.

Bass N.M., Kirsch R.E., Tuff S.A., Marks I. and Saunders S.J. (1977) *Biochim. Biophys. Acta* 492, 163-168.

Batist G., Tulpurle A., Sinha B., Katki A.G., Meyers C.E. and Cowan K.H. (1986) *J. Biol. Chem.* 261, 15544-15549.

Beatty P.W. and Reed D.J. (1980) *Arch. Biochem. Biophys.* 204, 80-87.

Beckett G.J. and Hayes J.D. (1993) *Adv. Clin. Chem.* 30, 281-300.

Belinsky M. and Jaiswal A.K. (1993) *Cancer Metastasis Rev.* 12, 103-117.

Benson A.M., Talalay P., Keen J.H. and Jakoby W.B. (1977) *Proc. Natl. Acad. Sci. USA.* 47, 158-162.

Bird R.P., Hung S.S.O., Hadley M. and Draper H.H. (1983) *Anal. Biochem.* 128, 240-244.

Booth J., Boyland E. and Sim P. (1961) *Biochem. J.* 79, 516-524.

Borroz K.I., Buetler T.M. and Eaton D.L. (1994) *Toxicol. Appl. Pharmacol.* 126, 150-155.

Bray T.M. and Taylor C.G. (1993) *Can. J. Physiol. Pharmacol.* 71, 746-751.

Brown L.A.S., Bai C. and Jones D.P. (1992) *Am. J. Physiol.* 262, L305-L312.

Brucchausen F., Wurm G. and Just I. (1988) in *Cellular Antioxidant Defense Mechanisms* (Chow C.K. ed.) Vol.1, pp. 117-137, CRC Press, Boca Raton, Florida.

Bucher J.R., Tien M. and Aust S.D. (1983) *Biochem. Biophys. Res. Commun.* 111, 777-784.

Burdon R.H. (1992) *Proc. Roy. Soc.* 99B, 169-176.

Burgess J.R., Hong Y., Chang M., Rao M.K., Tu C.-P.D. and Reddy C.C. (1987) *Biochem. Biophys. Res. Commun.* 142,441-447.

Burton G.W. and Ingold K.U. (1989) *Ann. N. Y. Acad. Sci.* 570, 7-31.

Cadenas E. (1984) *Photochem. Photobiol.* 40, 823-830.

Cagen L.M., Pisano J.J., Ketley J.N., Habig W.H. and Jakoby W.B. (1975) *Biochim. Biophys. Acta* 398, 205-208.

Carlberg I. and Mannervik B. (1985) *Methods Enzymol.* 113, 484-490.

Carmichael J., Forrester L.M., Lewis A.D., Hayes J.D., Hayes P.C. and Wolf C.R. (1988) *Carcinogenisis* 9, 1617-1621.

Carpenter M.P. (1981) *Fed. Proc.* 40, 189.

Carrillo M.-C., Nokubo M., Kitani K., Satoh K. and Sato K. (1991) *Biochim. Biophys. Acta* 1077, 325-331.

Chan A.C., Allen C.E. and Hegarty P.V.J. (1980) *J. Nutr.* 110, 66-73.

Chang M., Burgess J.R., Scholz R.W. and Reddy C.C. (1990) *J. Biol. Chem.* 265, 5418-5423.

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

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

Charles Kumar T., Veera Reddy K., Prasad M., Thyagaraju K. and Reddanna P. (1992) *Mol. Cell. Biochem.* 111, 109-115.

Chasseaud L.F. (1979) *Adv. Cancer Res.* 29, 175-274.

Chow C.K. and Tappel A.L. (1974) *J. Nutr.* 104, 444-451.

Christ -Hazelhof E., Nugteren D.H. and Van Drop D.A. (1976) *Biochim. Biophys. Acta* 450, 450-461.

Christensen M.J., Nelson B.L. and Wray C.D. (1994) *Biochem. Biophys. Res. Commun.* 202, 271-277.

Chu F.F., Dorshow J.H. and Esworthy R.S. (1993) *J. Biol. Chem.* 268, 2571-2576.

Clashen P.C., Moison R.M.W., Holtzer C.A.Z. and Berger H.M. (1992) *Pediatric Res.* 32, 399-401.

Coles B. and Ketterer B. (1990) *Crit. Rev. Biochem. Mol. Biol.* 25, 47-70.

Cooper D.R. and Carpenter M.P. (1987) *Biochem. J.* 241, 847-855.

Cossar D., Bell J., Strange R., Jones M., Sandison A. and Hume R. (1990) *Bochim. Biophys. Acta* 1037, 221-226.

Coursin D.B., Cihla H.P., Oberley T.D., and Oberley L.W. (1992) *Am. J. Physiol.* 263, L679- L691.

Crapo J.D., Barry B.E., Foscue H.A and Shelburne J. (1980a) *Am. Rev. Respir. Dis.* 122, 123-143.

Crapo J.D., Barry B.E., Foscue H.A. and Shelburne J. (1980b) *Am. Res. Environ. Exercise Physiol.* 44, 364-369.

Crintion R.A.P. and O'Connor J.P. (1978) *Irish Vet. J.* 32, 81-86.

Cucurou C, Battioni P., Daniel R. and Mansuy D. (1991) *Biochim. Biophys. Acta* 1081,99-105.

Dahinden C.A., Clancy R.M., Gross M., Chiller J.M. and Hugli T.E. (1985) *Proc. Natl. Acad. Sci. USA* 82, 6632-6636.

Dahlen S-E., Hedqvist P., Hammarstrom S. and Samuelsson B. (1980) *Nature* 288, 484-486.

DeLeve L.D. and Kaplowytz N. (1990) *Semin. Liver Dis.* 10, 251-266.

Deneke S.M. and Fanburg B.L. (1989) *Am. J. Physiol.* 257, L163-L173.

Derbel M., Igarashi T. and Satoh T. (1993) Arch. Biochem. Biophys. 1158, 175-180.

Diplock A.T. (1985) (ed.) in *Fat Soluble Vitamins, their Biochemistry and Applications*, pp. 319, Heineman, London.

Elasayed N.M., Kass R., Mustafa M.G., Hacker A.D., Ospital J.J., Chow C.K. and Cross C.E. (1988) *Drug Nutr. Interact.* 5, 373-386.

Esterbauer H., Schaur R.J. and Zollner H. (1991) *Free Rod. Biol. Med.* 11, 81-128.

Esterbauer H., Zollner H. and Schaur R.J. (1990) in *Membrane Lipid Oxidation* (Vigo-Pelfrey C. ed.) pp. 239-267, CRC Press, Florida.

Esworthy R.S., Chu F.F., Paxton R.J., Akman S. and Doroshow J.H. (1991) *Arch. Biochem. Biophys.* 286, 330-336.

Fiske C.H.and Subbarow Y. (1925) *J. Biol. Chem.* 66, 375-400.

Flohe L., Gunzler W.A. and Schock H.H. (1973) *FEBS Lett.* 32, 132-134.

Forman H.J. and Thomas M.J. (1986) *Ann. Rev. Physiol.* 48, 669-680.

Forman H.J., Rotman E.I. and Fisher A.B. (1983) *Lab. Invest.* 49, 148-153.

Freeman B.A. and Crapo J.D. (1982) *Lab. Invest.* 47, 412-426.

Fridovich I. (1989) *J. Biol. Chem.* 264, 7761-7764.

Gershman R., Gilbert D.L., Nye S.W., Dwyer P. and Fenn W.O. (1954) *Science* 119, 623-626.

Gey K.F., Brubacher G.B. and Stahelin H.B. (1987) *Am. J.Clin. Nutr.* 45, 1368-1377.

Gilbert H.G. (1984) *Methods Enzymol.* 107, 330-351.

Gutteridge J.M.C. and Halliwell B. (1990) *TIBS* 15, 129-135.

Habig W.H. and Jakoby W.B. (1981) *Methods Enzymol.* 77, 398-405.

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

Hafeman D.G. and Hoekstra W.G. (1977) *J. Nutr.* 107, 666-672.

Halliwell B.H. (1990) *Free Rad. Res. Commun.* 9, 1-32.

Halliwell B.H. (1994) *Nutr. Rev.* 52, 253-265.

Halliwell B.H. and Chirico S. (1993) *Am J.Clin. Nutr.* 57, 715S-725S.

Halliwell B.H. and Gutteridge J.M.C. (1989) in *Free Radicals in Biology and Medicine*. 2nd ed. Clarendon Press, Oxford, England.

Hamberg M, Svensson J., Wakobayashi T. and Samuelsson B. (1974) *Proc. Natl. Acad. Sci. USA*. 71, 345-349.

Hammarstrom S. and Samuelsson B. (1980) *Biochem. Biophys. Res. Commun.* 92, 946-953.

Hammond B. and Hess M.L. (1985) *J. Am. Coll. Cardiol.* 6, 215-220.

Harman D. (1956) *J. Geront.* 11, 298-300.

Hatayama C, Satoh K. and Sato K. (1986) *Biochem. Biophys. Res. Commun.* 140, 581-588.

Hawker F.H., Ward H.E., Stewart P.M., Wynne L.A. and Snitch P.J. (1993) *Eur. Respir. J.* 6, 1317-1323.

Hayes J.D. (1986) *Biochem. J.* 233, 789-798.

Hayes J.D. (1988) *Biochem. J.* 255, 913-922.

Hayes J.D. and Mantle T.J. (1986) *Biochem. J.* 237, 779-788.

Hayes J.D. and Pulford D.J. (1995; *Crit. Rev. Biochem. Mol. Biol.* 30, 445-600.

Hayes J.D., Judah D.J. and Neal G.E. (1993) *Cancer Res.* 53, 3887-3894.

Hayes J.D., Kerr L.A., Harrison D.J., Cronshaw A.D., Ross A.G. and Neal G.E. (1990) *Biochem. J.* 268, 295-302.

Hendrich S., Krueger S.K., Chen H.-W. and Cook L. (1991) *Prost. Leuk. Essent. Fatty acids* 42, 45-50.

Hill K.E. and Burk R.F. (1982) *J. Biol. Chem.* 257, 10668-10672.

Hill K.E., Burk R.F. and Lane J.M.(1987) *J. Nutr.* 117, 99-104.

Hinchman C.A. and Ballatori N. (1994) *J. Toxicol. Environ. Health* 41, 387-409.

Hiratsuka A., Sebata N., Kawashima K., Okuda H., Ogura K., Watanabe T., Satoh K., Hatayama I., Tsuchida S., Ishikawa T. and Sato K. (1990) *J. Biol. Chem.* 265, 11973-11981.

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

Hope W.C., Dalton C, Machlin L.F., Filiposki R.J. and Vane F.M. (1975) *Prostaglandins* 10, 557-578.

Horwitt M.K. (1986) *Am. J. Clin. Nutr.* 44, 973-985.

Hwang C, Sinskey A.J. and Lodish H.F. (1992) *Science* 257, 1496-1502.

Igarashi T., Irokawa N. and Ono S. (1987) *Xenobiotica* 17, 127-137.

Igarashi T., Satoh T., Hoshi K., Ueno K. and Kitagawa H. (1982) *Life Sci.* 31, 2655-2665.

Ip C. and Ganther H. (1990) *Cancer Res.* 50, 1206-1211.

Ip C. and Hayes C. (1989) *Carcinogenesis* 10, 921-925.

Jaeckson R.M. and Veal C.F. (1990) *Am. J. Physiol.* 259, H518-524.

Jain A., Martensson J., Mehta T., Krauss A.N., Auld P.A.M. and Meister A. (1992) *Proc. Natl. Acad. Sci. USA.* 89, 5093-5097.

Jakobson I., Warholm M. and Mannervik (1979) *Biochem. J.* 177, 861-868.

Jakoby W.B. and Habig W.H. (1980) in *Enzymatic Basis of Detoxification,* (Jakoby, W.B. ed.) vol. 2, pp. 63-94, Acaddemic Press, New York.

Jamieson D., Chance B., Cadenas E. and Boveris A. (1986) *Annu. Rev. Physiol.* 48, 703-719.

Jayatilleke E. and Shaw S. (1993) *Anal. Biochem.* 214, 452-457.

Jenkinson S.G., Jordan J.M. and Duncan C.A. (1989) *Am. J. Physiol.* 257, L393-L398.

Jenkinson S.G., Spence T.H., Lawrence R.A., Hill K.E., Duncan C.A. and Johnson K.H. (1987) J. *Appl. Physiol.* 62, 55-60.

Ji L.L. and Fu R. (1992) *J. Appl. Physiol.* 72, 549-554.

Ji L.L., Stratman F.W. and Lardy H.A. (1988) *Arch. Biochem. Biophys.* 263, 150-160.

Ji L.L., Stratman F.W. and Lardy H.A. (1992a) *J. Am. Coll. Nutr.* 11, 79-86.

Ji X., Zhang P., Armstrong R.N. and Gilliland G.L. (1992b) *Biochemistry* 31, 10169-10184.

Johnson J.A., Finn K.A. and Siegel F.L. (1992) *Biochem. J.* 282, 279-289.

Johnson K.J., Fantone J.C., Kaplan J. and Ward P.A. (1981) *J. Clin. Invest.* 67, 983-993.

Kagan V.E., Spiricheu V.B. and Erin A.E.(1989) in *Nutrition in Exercise and Sport* (Hickson F.J. and Wolinsky I. eds) pp. 255-278, CRC Press,Boca Raton, Florida.

Kalinyak J.E. and Taylor J.M. (1982) *J. Biol. Chem.* 257, 523-530.

Kanter M.M.(1994) *Int. J. Sport Nutr.* 4, 205-220.

Kauf E., Janitzky E., Vogt L., Winnefeld K., Dawczynski H., Forberger M., Jahreis G, and Vogel H. (1995) *Med. Klin.* 90, 41-45.

Kensler T.W., Egner P.A., Davidson N.E., Roebuck B.D., Pikul A. and Groopman J.D. (1986) *Cancer Res.* 46, 3924-3931.

Ketterer B. and Christodoulides L.G. (1994) *Adv. Pharma.* 27, 37-69.

Ketterer B., Meyer D.J. and Clark A.G. (1988) in *Glutathione Conjugation, Mechanism and Biological Significance* (Sies H. and Ketterer B. eds.) pp. 74-135, Academic Press, London.

Kim Y.S. and Combs Jr. G.F. (1993) *Nutr. Res.* 13, 455-463.

Kitahara A., Satoh K., Nishimura K., Ishikawa T., Ruike K., Sato K., Tsuda H. and Ito N. (1984) *Cancer Res.* 44, 2698.

Koppenol W.H. (1990) in *Membrane Lipid Oxidation* (Vigo-Pelfrey C. ed.) pp. 1-13, CRC Press, FL.

Korpela H., Kumpulainen J., Luoma P.V., Arranto A.J. and Sotaniemi E. (1985) *Am. J. Clin. Nutr.* 42, 147-151.

Kretzchmar M. and Klinger W. (1990) *Exp. Pathol.* 36, 145-164.

Krinsky I.N. (1992) *Proc. Soc. Exp. Biol. Med.* 200, 248-254.

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

Lai H.-C.J., Li N.Q., Weiss M.H., Reddy *C.C.* and Tu C.-P.D. (1984) *J. Biol. Chem.* 259, 5536-5542.

Lands W.E.M., Kulmacz R.J. and Marshall P.J. (1984) in *Free Radicals in Biology* (Pryor W.A. ed.) Vol. VI, pp. 39-61, Academic Press, New York.

Landstein R., Epp O., Huber R. and Wendel A. (1981) *IBID* 33-43.

Landstein R., Epp O., Bartels K., Jones A., Huber R. and Wendel A. (1979) *J. Mol. Biol.* 134, 199-218.

Larsson A., Orrenius S., Holmgren A. and Mannervik B. (1983) in *Functions of Glutathione- Biochemical, Physiological, Toxicological and Clinical Aspects,* Raven Press, New York.

Lauterburg B.H., Adams J.D. and Mitchell J.R. (1984) *Hepatology* 4, 586-590.

Lawrence R.A. and Burke R.F. (1976) *Biochem. Biophys. Res. Commun.* 71, 952-958.

Lawrence R.A., Parkhill L.K. and Burk R.F. (1978) *J. Nutr.* 108, 981-987.

Lee C.W., Lewis R.A., Corey E.J. and Austen K.F. (1983) *Immunology* 48, 27-35.

Lei D.N., Wang L.Q., Ruebner B.H., Msieh D.P., Wu B.F., Zhu C.R. and Du M.J. (1990) *Biomed. Environ. Sci.* 3, 65-80.

Lomnitski L., Bergman M., Schion I. and Grossman S. (1991) *Biochim. Biophys. Acta* 1082, 101-107.

Lowry O.H., Roserbrough N.J., Farr A.L. and Randall R.L. (1951) J. *Biol. Chem.* 193, 265-275.

Luoma P.V., Nayha S., Sikkila K. and Hassi J. (1995) *J. Intern. Med.* 237, 49-54.

Maddipati K.R. and Marnett L.J. (1987) *J. Biol. Chem.* 262, 17398-17403.

Mannervik B. (1986) *Chemica Scripta* 26B, 281-284.

Mannervik B. and Danielson U.H. (1988) *Crit. Rev. Biochem. Mol. Biol.* 23, 283-337.

Mannervik B., Alin P., Guthenberg C, Jensson H., Tahir M.K., Warholm M. and Jornvall H. (1985) *Proc. Natl. Acad. Sci. USA.* 82, 7202-7206.

Martensson J., Jain A., Frayer W. and Meister A. (1989) *Proc. Natl. Acad. Sci. USA.* 86, 5296-5300.

Martensson J., Jain A., Stole E., Frayer W., Auld P.A.M. and Meister A. (1991) *Proc. Natl. Acad Sci. USA.* 88, 9360-9364.

McCay P.B. and King M. (1980) in *Vitamin E: A Comprehensive Treatise* (Machlin W. ed.) pp. 289-317, Marcel Dekkar, New York.

McCord J.M. and Fridovich I. (1968) *J. Biol. Chem.* 244, 6049-6055.

McIntyre T.M. and Curthoys N.P. (1980) *Int. J. Biochem.* 12, 545-551.

Mehlert A. and Diplock AT. (1985) *Biochem. J.* 227, 823-831.

Meister A. (1991) *Pharmacol. Ther.* 51, 155-194.

Meister A. and Anderson M.E. (1983) *Annu. Rev. Biochem.* 52, 711-760.

Meydani S.N., Shapiro A.C., Meydani M. and Blumberg J.B. (1992) *J. Nutr.* 122, 1627-1633.

Meyer D.J. (1993) *Xenobiotica* 22, 823-834.

Meyer D.J., Harris J.M., Gilmore K.S., Coles B., Kensler T.W. and Ketterer B. (1993) *Carcinogenesis* 14, 567-572.

Moncada S., Palmer R.M.J. and Higgs E.A. (1991) *Pharmacol. Rev.* 43, 109-142.

Murata T., Hatayama I., Satoh K., Tsuchida S. and Sato K. (1990) *Biochem. Biophys. Res. Commun.* 171, 845-851.

Niki E., Tsuchiya J., Tanimura R. and Kamiya Y. (1982) *Chem. Lett.* 789-792.

Oakley B.R., Kirsch D.R. and Morris R.N. (1980) *Arch. Biochem. Biophys.* 105, 361-363.

Ogorachi T., Utihara M. and Narumiya S. (1987) *J. Neurochem.* 48, 900-909.

Ostero and Prellwitz W. (1990) *Biol. Trace Elem. Res.* 24, 91.

Ostlund F., Meyer D.J., Coles B., Southan C, Aitken A., Johnson P.J. and Ketterer B. (1987) *Biochem. J.* 245, 423-428.

Panganamala R.U. and Cornwell D.G. (1980) *Ann. N. Y. Acad. Sci.* 393, 376-391.

Panus P.C., Shearer J. and Freeman B.A. (1988) *Exp. Lung Res.* 14, 959-976.

Panush R.S. and Delafuente J.C. (1985) *World Rev. Nutr. Diet* 45, 97-132.

Pemble S.E., Taylor J.B., Craig R.K. and Ketterer B. (1986) *Biochem. J.* 238, 373-378.

Penrose J.F., Gagnon L., Goppelt-Struebe M., Meyers P., Lam B.K., Jack R.M., Austen K.F. and Soberman R.J. (1992) *Proc. Natl. Acad. Sci. USA.* 89, 11603-11606.

Pickett C.B., Telakowski-Hopkins C.A., Ding G.J.-F., Algenbright L. and Lu A.Y.H. (1984) *J. Biol. Chem.* 259, 5182-5188.

Piper P.J. (1984) *Physiol. Rev.* 64, 744-761.

Potter D.W. and Tran T-B. (1993) *Toxicol. Appl. Pharmacol* 120, 186-192.

Primiano T. and Novak R.F. (1992) *Toxicol. Appl. Pharmacol.* 113, 64-73.

Reddanna P., Maddipati K.R. and Reddy C.C. (1985) *FEBS Lett.* 193, 39-43.

Reddanna P., Whelan J. and Reddy C.C. (1988) *Arm. N. Y Acad. Sci.* 524, 391-392.

Reddanna P., Whelan J., Burgess J.R. Eskew M.L., Hildenbrandt G., Zarkower A., Scholz R.W. and Reddy C.C. (1989) *Ann. N. Y Acad. Sci.* 570, 136-145.

Reddy C.C., Whelan J. and Scholz R.W. (1988) in *Cellular Antioxidant Defense Mechanisms* (Chow C.K. ed.) Vol.1, pp. 139-149, CRC Press, Florida.

Reddy C.C, Burgess J.R., Gong Z.Z., Massaro E.J. and Tu C.-P.D. (1983) *Arch. Biochem. Biophys.* 224, 87-101.

Reddy C.C, Grasso T.C., Scholz R.W., Labosh T.J., Thomas C E. and Massaro E.J. (1982) *Prostaglandins and Cancer Intl. Conference* (Powells T.J., Ramwell P.W., Honn K.V. and Bockman R.S. eds.) pp. 149-154, Alan R. Liss, New York.

Reddy C.C, Tu C.-P.D., Burgess J.R., Ho C-Y., Scholz R.W. and Massaro E.J (1981) *Biochem. Biophys. Res. Commun.* 101,970-978.

Reed D.J. (1990) *Annu. Rev. Pharmacol. Toxicol.* 30, 603-631.

Reuter A. and Klinger W. (1992) *Exp. Toxicol. Pathol.* 44, 339-343.

Richman P.G. and Meister A. (1975) *J. Biol. Chem.* 250, 1422-1426.

Robertson I.G.C., Jensson H., Mannervik B. and Jernstrom (1986), *Carcinogenesis* 7, 295.

Rokizki L., Logemann E., Sagredos A.N., Murphy M., Wetzel-Roth W. and Keul J. (1994) *Acta Physiol. Scand.* 151, 149-158.

Rotruck J.T., Pope A.L., Ganther H.E., Swasson A.B., Hafeman D. and Hoekstra W.G. (1973) *Science* 179, 588-590.

Rushmore T.H., Morton M.R. and Pickett C.B. (1991) J. *Biol. Chem.* 266, 11632-11639.

Rushmore T.H., Nguyen T. and Pickett C.B. (1993) in *Structure and Function of Glutathione S-Transferase* (Tew K.D., Pickett C.B., Mantle T.J., Mannervik B. and Hayes J.D. eds.) pp. 119-128, CRC Press, Boca Raton, Florida.

Rushmore T.H., King R.G., Paulson K.E. and Pickett C.B. (1990) *Proc. Natl. Acad. Sci. USA.* 87, 3826-3830.

Samuelsson B. (1983) *Science* 220, 568-575.

Sato K. (1989) *Adv. Cancer. Res.* 52, 205.

Satoh T., Takenaga M., Kitagawa H. and Itoh S. (1980) *Res. Comm. Chem. Path. Pharmacol.* 30, 151-161.

Scandalios J.G. (1993) *Plant Physiol.* 101, 7-12.

Schwartz K. and Foltz C.M. (1957) *J. Am. Chem. Soc.* 79, 3292-3293.

Schwartz L.H., Urban T. and Hercberg S. (1994) *Presse-Med.* 23, 1826-1830.

Scott M.L. (1978) in *The Fat-Soluble Vitamins* (DeLuka H.F. ed.) pp. 133-210, Plenum Press, New York.

Seidegard J. and Pero R.W. (1985) *Human Genet.* 69, 66-68.

Sen C.K., Marin E., Kretzchmar M. and Hanninen 0.(1992) *J. Appl. Physiol.* 73, 1265-1272.

Simons P.C. and Vander Jagt L. (1977) *Methods Enzymol.* 77, 235-237.

Singhal S.S., Saxena M., Ahmad H., Awasthi S., Haque A. and Awasthi Y.C. (1992) *Arch. Biochem. Biophys.* 299, 232-241.

Sjodin B., Hellsten-Westng Y. and Apple F.S. (1990) *Sports Med.* 10, 236-254.

Smedegard G., Hedqvist P., Dahlen S-E., Revenas B., Hammarstrom S. and Samuelsson B. (1982) *Nature* 295, 327-334.

Smith L.J. and Anderson J. (1992) *Am. Rev. Respir. Dis.* 146, 1452-1457.

Smith C.M., Kelsey K.T., Wiencke J.K., Leyden K., Levin S. and Christiani D.C. (1994) *Biomarkers Prev.* 3,471-477.

Soderstrom M., Hammarstrom S. and Mannervik B. (1988) *Biochem. J.* 250, 713-718.

Spencer S.R., Xue L., Klenz E.M. and Talalay P. (1991) *Biochem. J.* 273, 711-717.

Stenberg G., Rodderstrom M., Engstrom A., Pemble S.E. and Mannervik B. (1992) *Biochem. J.* 284, 313-319.

Stuart M. (1982) *Ann. N. Y. Acad. Sci.* 293, 277

Sunde R.A. and Hoekstra W.G. (1980) *Nutr. Rev.* 38, 265-273.

Svovoda D.J. and Higginson J. (1963) *Am. J. Pathol.* 43, 477-495.

Tahir M.K., Guthenberg C. and Mannervik B. (1989) *Biochem. J.* 257, 215-220.

Talalay P. and Spencer S.R. (1990) in *Glutathione S-Transferase and Drug Resistance* (Hayes J.D., Pickett C.B. and Mantle T.J. eds.) pp. 176-185, Taylor and Francis, London.

Tan K.H., Meyer D.J., Belin J. and Ketterer B. (1984) *Biochem. J.* 220, 243-252.

Tan K.H., Meyer D.J., Gillies N. and Ketterer B. (1988; *Biochem. J.* 254, 841-845.

Tappel A.L. (1962) in *Vitamins and Hormones* pp. 493-510, Acadmic Press, Orlando, Florida.

Tappel A.L. (1980) *Ann. N. Y Acad. Sci.* 355, 212-216.

Tappel A.L., Hawkes W.C., Wilhelmsen E.C. and Monsenbocker M.A. (1984) *Methods Enzymol.* 107, 602-619.

Tate S.S., Thompson G.A. and Meister A. (1976) in *Glutathione Metabolism and Function* (Arias I.M. and Jakoby W.B. eds.) pp. 45-55, Raven Press, New York.

Tateishi N., Higashi T., Naruse A., Nakashima K., Shiozaki H. and Sakamoto Y. (1977) *J. Nutr.* 107, 51-60.

Taylor S.L., Lamden M.P. and Tappel A.L. (1976) *Lipids* 11, 530-538.

Thyagaraju K., Burgess J.R., Hong Y., Reddanna P., Tu C.-P.D. and Reddy C.C. (1986a) *Fed. Proceed AS,* 1000.

Thyagaraju K., Burgess J.R., Reddanna P., Hong Y., Tu C.-P.D. and Reddy C.C. (1986b) *Fed. Proceed.* 45, 1652.

Tiidus P.M., Behrens W.A., Madere R., Kim J.J. and Houston M.E. (1993) *Nutr. Res.* 13, 219-224.

Tipping E. and Ketterer B. (1981) *Biochem. J.* 195, 441-442.

Towbin H., Staehelin T. and Gordon J. (1979) *Proc. Natl. Acad. Sci. USA.* 76, 4350-4354.

Tsuchida S. and Sato K. (1990) *Biochem. J.* 266, 461-465.

Tsuchida S. and Sato K. (1992) *Crit. Rev. Biochem Mol. Biol.* 27, 337-384.

Tsuchida S., Izumi T., Shimizu T., Ishikawa T., Hatayama I., Satoh K. and Sato K. (1987) *Eur. J. Biochem.* 170, 159-164.

Tu C.-P.D. and Reddy C.C. (1985) J. *Biol. Chem.* 260, 9961-9964.

Tu C.-P.D., Weiss M.J., Li N.Q. and Reddy C.C. (1983) *J. Biol. Chem.* 258, 4659-4662.

Urcini F., Maiorino M., Valente M., Ferri L. and Gregolin C. (1982) *Biochim. Biophys.Acta* 710, 197-211.

Van Vleet J.F. (1982) *Am. J. Vet. Res.* 43, 851-857.

Vander Jagt G.L., Hunsaker L.A., Garcia K.B. and Royer R.E. (1985) *J. Biol. Chem.* 260, 11603-11610.

Van-Poppel G., Kardinnal A., Prince H. and Kok F.J. (1994) *Annu. Med.* 26, 429-434.

Veera Reddy K., Anuradha D., Charles Kumar T. and Reddanna P. (1995) *Arch. Biochem. Biophys.* 323, 6-10.

Vos R.E.M. and Van Bladderin P.J. (1990) *Chem. Biol. Interaction* 75, 241-265.

Warburg C. and Christian W. (1941) *Biochem. Z* 310, 402.

Waxman D.J., Sundseth S.S., Srivastava P.K. and Lapenson D.P. (1992) *Cancer Res.* 52, 5797-5802.

Whanger P.D (1989) *J. Nutr.* 119, 1236-1239.

Whelan J., Reddanna P., Burgess J.R. and Reddy C.C. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 45, 1935.

Yamamoto S. (1982) *Methods Enzymol.* 186, 55-60.

Zhang L. (1994) *Carcinogenesis* 15, 105-109.