METHODOLOGY
2.1.0.0. Materials

Arachidonic acid, \( \text{PGD}_2, \text{PGE}_2, \text{PGF}_{2\alpha}, \text{GSH}, \text{GSSG}, \text{vitamin E (d-\alpha-tocopherol)} \), \( \text{NADPH} \), \( \text{TBA} \) (thiobarbituric acid), Alkaline phosphatase, \( \text{Freund’s} \) complete and incomplete adjuvants, \( \text{NBT} \) (nitroblue tetrazolium), \( \text{BCIP} \) (5-bromo-4-chloro-3-indoyl phosphate), \( \text{BHT} \) (butylated hydroxytoluene) and \( \text{DAN} \) (2,3-diaminonaphthalene) were purchased from Sigma Chemicals Company, St. Louis, USA. \( ^3\text{H} \) 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 \( \text{acetonitrile} \), methanol. acetic acid, trifluoroacetic acid, triethylamine were purchased from SD fine Chemicals India Ltd., and \textbf{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 \( \text{Se/Kg diet} \), as sodium selenite, and/or with 100 IU vitamin E/Kg diet, as d \( \alpha \)-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 \textit{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 perfusion 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

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>AMOUNT %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin free casein</td>
<td>20</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>63.7</td>
</tr>
<tr>
<td>Mineral mixture*</td>
<td>4</td>
</tr>
<tr>
<td>Vitamin mixture**</td>
<td>1</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
</tr>
<tr>
<td>Vitamin free corn oil</td>
<td>5</td>
</tr>
</tbody>
</table>

* 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 α-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 Shimpack 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 HCl 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% KCl and 0.5 mM BHA (in methanol) was added. To the sample 3 mL of 0.44 mol of H₃PO₄/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 C₁₈ Bondapak (3.9 X 300 mm) column and eluted with 50 mM KH₂PO₄, 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 C18 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 MgCl₂, 5 mM disodium ATP, 2 mM EDTA, 10 mM sodium L-glutamate, 10 mM L-a-aminobutyrate, 20 mM MgCl₂, 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, iBondapak (Waters)
- Solvents: 13% Methanol in 25 mM NaH_2PO_4; 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 MgCl₂, 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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Buffer pH</th>
<th>[Substrate] mM</th>
<th>[GSH] mM</th>
<th>Absorbance nm</th>
<th>Δε</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-Dichloro-4-nitrobenzene</td>
<td>7.5</td>
<td>1.0</td>
<td>5.0</td>
<td>345</td>
<td>8.5</td>
</tr>
<tr>
<td>1-Chloro-2,4-dinitrobenzene</td>
<td>6.5</td>
<td>1.0</td>
<td>1.0</td>
<td>340</td>
<td>9.6</td>
</tr>
<tr>
<td>Δ⁵-Androstene-3,17-dione</td>
<td>8.5</td>
<td>0.068</td>
<td>0.1</td>
<td>248</td>
<td>16.3</td>
</tr>
<tr>
<td>1,2-Epoxy-3-((p-nitrophenoxy)-propane</td>
<td>6.5</td>
<td>0.5</td>
<td>5.0</td>
<td>360</td>
<td>0.5</td>
</tr>
<tr>
<td>4-Nitropyridine-N-oxide</td>
<td>7.0</td>
<td>0.2</td>
<td>5.0</td>
<td>295</td>
<td>7.0</td>
</tr>
<tr>
<td>p-Nitrobenzyl chloride</td>
<td>6.5</td>
<td>1.0</td>
<td>5.0</td>
<td>310</td>
<td>1.9</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>6.5</td>
<td>0.2</td>
<td>0.25</td>
<td>270</td>
<td>5.0</td>
</tr>
<tr>
<td>p-Nitrophenyl acetate</td>
<td>7.0</td>
<td>0.2</td>
<td>0.5</td>
<td>400</td>
<td>8.79</td>
</tr>
<tr>
<td>Bromosulphthalein</td>
<td>7.5</td>
<td>0.03</td>
<td>5.0</td>
<td>330</td>
<td>4.5</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>7.0</td>
<td>0.03</td>
<td>1.0</td>
<td>340</td>
<td>6.2</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>7.0</td>
<td>0.25</td>
<td>1.0</td>
<td>340</td>
<td>6.2</td>
</tr>
<tr>
<td>t-Butyl hydroperoxide</td>
<td>7.0</td>
<td>1.5</td>
<td>1.0</td>
<td>340</td>
<td>6.2</td>
</tr>
<tr>
<td>13-HODE</td>
<td>7.0</td>
<td>0.03</td>
<td>1.0</td>
<td>340</td>
<td>6.2</td>
</tr>
<tr>
<td>15-HPETE</td>
<td>7.0</td>
<td>0.03</td>
<td>1.0</td>
<td>340</td>
<td>6.2</td>
</tr>
</tbody>
</table>
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°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 purification. 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 KCl and 75-200 mM KCl 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 KCl and 100 mL of buffer B containing 200 mM KCl.

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 KCl 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 u Bondapak C18 (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% acetonitrile (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 subunit 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 14C 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 HCl and reaction products were extracted twice with 3 volumes of chloroform. Organic phases were pooled and evaporated under N₂ 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 LTC₄ 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 LTA₄ Me (final concentration 30 µM) test tube, which was previously evaporated to dryness under N₂ 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 C₁₈ 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 LTA₄ Me products were separated on HPLC (Shimadzu LC 6AD) by the method of Chang et al. (1987b) using C₁₈ 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 LTC₄ Me formed was quantified basing on HPLC integration data and molar extinction coefficient of LTC₄ (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',N'-methylene-bis-acrylamide.

Samples were boiled at 100°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 Y\(a_1\) and \(a_2\)), Yb (pooled Y\(b_1\) and Y\(b_2\)) and Yc recovered from RP-HPLC. Similarly for lung tissue also antibodies were raised against lung affinity purified GSTs and Yc, Yb (pooled Y\(b_1\) and Y\(b_2\)), 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 1 hr. 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 min X 3 times each and finally developed with buffer E consisting of 100 mM Tris-HCl; pH 9.5, 100 mM NaCl, 5 mM MgCl₂, 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.