SHORT TERM AND LONG TERM EFFECTS OF CURCUMIN ON ACTIVITIES OF GLUTATHIONE S-TRANSFERASE AND CYTOCHROME P450 IN LIVERS OF RATS

SALEHA HUSAIN

Short Term and Long Term Effects of Curcumin on Activities of Glutathione S-Transferase and Cytochrome P450 in Livers of Rats

by

Saleha Husain, BS (Biology)

A report submitted to the School of Graduate Studies

in partial fulfilment of the

requirements for the degree of

Master of Environmental Science

Department of Environmental Science

Memorial University of Newfoundland

April 1997

St. John's Newfoundland Canada

ABSTRACT

Curcumin, a yellow compound naturally occurring in the plant *Curcuma longa Linn*, was studied for its in vivo short term and long term effects on the activities of some glutathione S-transferase (GST) and cytochrome P450 isozymes in livers of rats. The rats were fed 1% curcumin in an AIN 76 diet which contained 15% protein for a duration of approximately two weeks for short term studies and for a duration of approximately six weeks for long term studies. Glutathione S-transferase (GST) activities were measured in liver cytosols by using 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) as substrates. Cytochrome P450 1A1/2 and 2B1 activities were measured as ethoxyresorufin deethylation (EROD) and pentoxyresorufin depentylation (PROD) respectively in liver microsomes. Curcumin treatment was found to significantly inhibit GST activities in the long term studies as compared to controls when CDNB and DCNB were used as substrates. There was no significant inhibition of glutathione S-transferase (GST) activities by curcumin in the short term studies, as compared to controls. Curcumin treatment in both the short term and long term studies showed no significant effects on cytochrome P450 1A1/2 and 2B1 activities, as compared to controls. In vitro, curcumin was found to be a potent inhibitor of cytochrome P450 1A1/2 and 2B1 activities. These results suggest that short term administration of curcumin can lead to no significant effects on glutathione S-transferase (GST) and cytochrome P450 activities. However, long term administration of curcumin can lead to inhibition of

glutathione S-transferase activities (GST) but no significant effects on cytochrome P450 activities.

ACKNOWLEDGEMENTS

I would like to express deep appreciation and thank to my supervisor, Dr. Anver D. Rahimtula, for his excellent guidance, kind help, advice and encouragement throughout the course of this work.

I would also like to thank Marie Codner for co-supervising my work in the laboratory. I am deeply grateful to Misuk Yun, Sandy Crowley, Jiggs Diegor and friends in USA and Saudi Arabia for giving me constant encouragement and advice throughout the course of this work.

Finally, I would like to thank my parents and brothers for giving me constant encouragement, academic guidance, great love and care in this world.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES AND GRAPH

I. Introduction

1.1 Curcumin: Its occurrence

Curcumin is a naturally occurring yellow compound which is found in the rhizomes of the plant *Curcuma longa Linn* (family Zingiberaceae). This plant is commonly known as turmeric. Turmeric, as a spice, is widely used in food for flavoring and coloring. In medicine, turmeric is used in drugs (Govindarajan, 1980). Turmeric has been used in traditional medicine (known as ayuverdic medicine in India) for more than 2000 years and its broad spectrum of biological activities are mainly due to the curcuminoid contents (Tennesen, 1992). Curcuminoids are compounds which are structurally related to curcumin but are not necessarily functionally related to curcumin. In the rhizomes of the plant *Curcuma longa Linn*, beside curcumin, two other curcuminoids are found: (4-hydroxy-3-methoxycinnamoyl)methane and bis-(4-hydroxycinnamoyl)methane. Oshawa *et al.*(1995) extracted curcumin along with the curcuminoids from the rhizomes of *Curcuma longa Linn* after purification by preparative silica gel TLC (5% MeOH in $CHCl_3$). The yield was 76.0% curcumin, 19.8% (4-hydoxy-3-methoxycinnamoyl)methane and 8.6% bis-(4-hydroxycinnamoyl)methane.

1.2 Chemical properties of curcumin

Curcumin is the main coloring component and active agent in turmeric. The IUPAC name of curcumin is 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-

3,5-dione. Chemically, curcumin is commonly known as diferuloyl methane (Susan, 1992).

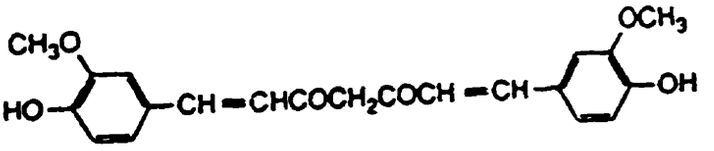


Figure 1: Chemical Structure of Curcumin

Curcumin exhibits anti-inflammatory properties. Several studies have been undertaken to evaluate the mechanisms of anti-inflammatory actions of curcumin (Tennesen, 1989; Tennesen and Greenhill, 1992). The results obtained indicate that curcumin, in solution, has a dual effect on oxygen radical reactions. Curcumin can act as a scavenger of hydroxyl radicals or catalyse the formation of hydroxyl radicals depending on the experimental conditions (Tennesen, 1993).

Curcumin can undergo photochemical degradation. Formation of a complex between curcumin and iron and reduction of Fe^{3+} to Fe^{2+} in the presence of curcumin have also been observed (Tennesen and Greenhill, 1992). The observed photoactivity of curcumin makes curcumin a potential photosensitizing drug.

Curcumin has structural similarities to ferulic acid which is a phenol found in plants. Ferulic acid is also known as an alkaline degradation product of curcumin (Tennesen and Karlsen, 1985). Plant phenols such as ferulic acid have been shown to

possess inhibitory properties towards glutathione S-transferases (GSTs) with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate (Das *et al.*, 1984). Curcumin is an α,β unsaturated carbonyl compound and is capable of undergoing Michael type addition reaction with glutathione both enzymatically and non-enzymatically (Susan and Rao, 1992).

1.3 Enzymatic properties of glutathione S-transferase

Glutathione S-tranferases (GSTs) are a family of enzymes that primarily catalyze the reaction of glutathione (GSH) with compounds that bear electrophillic centers such as CDNB and 1,2-dichloro-4-nitrobenzene (DCNB). CDNB and DCNB undergo nucleophillic displacement of the chloro substituent by GSH (Seis, 1988). GST-catalyzed reactions are generally detoxicating because GST is capable of inactivating a broad spectrum of foreign compounds (Dolphin *et al.*, 1989).

GST has affinity to bind to xenobiotics or endogenous hydrophobic compounds. In such cases GST is unable to conjugate the hydrophobic compound to GSH. Examples of endogenous compounds in the erythrocyte that bind to GST are bilirubin and heme (Dolphin *et al.*, 1989).

1.4 Enzymatic properties of cytochrome P450

Like GSTs, cytochrome P450s (P450s) are a family of enzymes which are involved in the metabolism of xenobiotics and can also act on a variety of compounds which are endogenous in mammalian systems. The P450 system involves two prosthetic groups,

heme and flavin, which can be limiting under certain conditions. The cofactors are oxygen and NADPH.

Specifically, microsomal P450s play primary roles in the oxidation of xenobiotic chemicals and steroids in the steroidic tissues. No cytosolic form of eukaryotic P450 has been found but a protein termed H450 has been found in beef and liver cytosols of rats. H450 has some properties very similar to other P450s (Dolphin *et al.*, 1989). The basic mechanisms of P450 oxidation reactions can be summarized as the initial abstraction of an electron or hydrogen atom followed by an "oxygen rebound" step (Seis, 1988). The effects of curcumin on the activities of rat liver microsomal P450, 1A1/2 and 2B1, can be measured by carrying out 7-ethoxyresorufin deethylation (EROD) and 7-pentoxyresorufin depentylation (PROD) reactions respectively in the presence of NADPH .

II. Definition of the problem

Curcumin is widely used in food for coloring and flavoring. Until now, no sufficient research has been published concerning the effects of curcumin on activities of GST and P450 which are two of the most important enzyme systems involved in the bioactivation and bioinactivation of xenobiotic compounds.

III. Objective of the study

- To investigate the effects of curcumin on:
- CDNB GSH conjugation activities in liver cytolosic GST of rats.

- DCNB GSH conjugation activities in liver cytosolic GST of rats.
- Cytochrome P450-dependent EROD activities in liver microsomes of rats.
- Cytochrome P450-dependent PROD activities in liver microsomes of rats; to be able to:

♦ Study the short term and long term effects of curcumin on activities of GST and cytochrome P450 in livers of rats.

IV. Experimental methods

The research work can be divided into seven phases. These seven phases of the experiment are outlined in figure 2. Phase I of this research project started on May 27, 1996. Phase VII of the research project was completed around the middle of August 1996.

Gloves and safety precautions were used all the time in phase I to phase VII in this research project. Care was taken to avoid contamination of samples and equipments.

4.1 Selection of rats

A total of 16 male Sprague-Darnley rats were selected for this research project. Eight rats were born later than the other eight rats. The younger rats were smaller in size than the older rats. The rats were caged as outlined in figure 3.

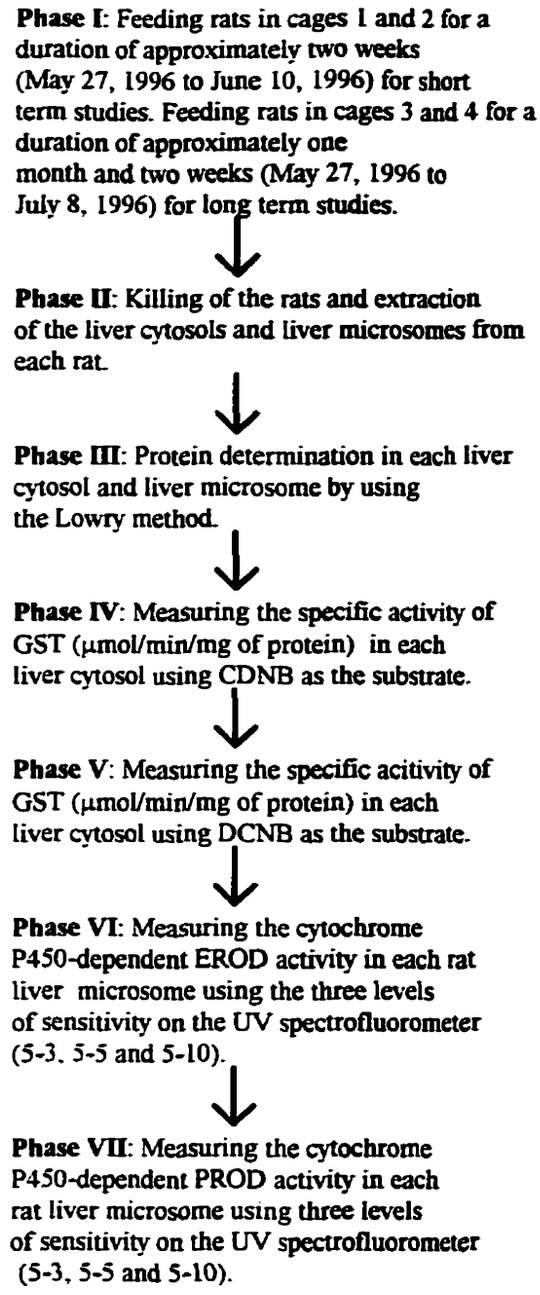


Figure 2: Outline of the different phases of the research project.

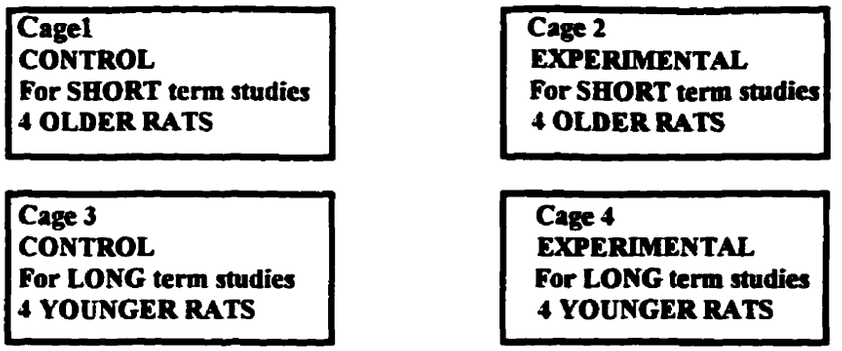


Figure 3: Grouping of the rats into cages for short term and long term studies.

The control and the experimental rats differed only in diet. All environmental conditions such as temperature and water supply were the same for the experimental and control rats.

4.2 Phase I: Feeding the rats

The rats in cages 1 and 3 (control) were fed AIN 76 Diet (contains 15% protein). The rats in cages 2 and 4 (experimental) were fed AIN 76 Diet (contains 15% protein) mixed with 1% curcumin. The rats in cages 1 to 4 were fed their respective diets until the day they were killed.

4.2.1 AIN 76 Diet (contains 15% protein)

Table 1 outlines the ingredients for making AIN 76 Diet (contains 15% protein).

Table 1: Ingredients for making 4 kg of AIN 76 Diet (contains 15% protein).

Ingredient	Quantity (grams)
AIN 76 Vitamin Mix	40
AIN 76 Mineral Mix	140
L-Methionine	6
Choline Bitartarate	4.8
Casein	594
Sucrose	2132
Corn Starch	680
Alphacel	200
Mazola Oil	200

The first four ingredients were mixed thoroughly first before adding the other components. The mixture was mixed thoroughly. The diet was stored in a plastic container in the cold room and fed daily to the control rats (cages 1 and 3).

4.2.2 1% curcumin diet

For 1% curcumin diet, 5 grams of curcumin was added to 500 grams of the prepared AIN 76 diet. The mixture was mixed thoroughly. This 1% curcumin diet was protected from light and was stored in a plastic container and kept in the cold room. It was fed daily to the experimental rats (cages 2 and 4).

4.3 Phase II: Killing the rats and extracting the liver cytosols and liver microsomes

4.3.1 Killing the rats

The rats in cages 1 and 2 were for short term studies. Two rats from cage 1 (control) and two rats from cage 2 (experimental) were killed on June 11, 1996. The

other two rats from cage 1 (control) and cage 2 (experimental) were killed on June 12, 1996.

The rats in cages 3 and 4 were for long term studies. Two rats from cage 3 (control) and two rats from cage 4 (experimental) were killed on July 9, 1996. The remaining two rats from cage 3 (control) and cage 4 (experimental) were killed on July 10, 1996.

4.3.1.1 Cervical dislocation

The rats were killed by the method of cervical dislocation. In this method, a rat was picked up firmly and immediately (but not tightly) by the anterior part of its tail and the head of the rat was banged against the table. Then immediately, the body of the rat was stretched away from its head. The body of the rat was cut open and the heart of the rat was attacked with a scissor to stop the heart functioning.

The method of cervical dislocation is suitable for killing rats when the focus is on the biochemical or chemical aspects of the digestive system. Other methods for killing rats, such as chemicals exposures to rats, are usually not suitable because the chemicals can react in the body of the rat and nullify the actual experimental results.

4.3.2 Extraction of the liver cytosols and liver microsomes

The following protocol was used for the extraction of the liver cytosols and liver microsomes from the killed rats:

(1) The body of the killed rat was cut . The liver was taken out and weighed.

(2) Then the liver was put in a beaker containing enough 0.1 M KP buffer (potassium phosphate buffer) at pH 7.4 to cover the liver.

(3) The liver was cut into small pieces using scissors.

(4) The buffer was discarded and fresh buffer was added about three times the weight of the liver.

(5) The liver was homogenized.

(6) The homogenate was poured into a 50ml centrifuge tube.

(7) The homogenates were centrifuged at 2000 rpm for five minutes.

(8) Then the homogenates were centrifuged at 10,000 rpm for another 12 to 15 minutes.

(9) Each supernatant from a liver homogenate was filtered through four layers of cheesecloth into a 25 ml centrifuge tube.

(10) The filtered supernatants were centrifuged at 37,000 rpm for 75 minutes.

(11) The supernatants from step 11 are the cytosols. The pellet below a supernatant is the microsomal fraction.The supernatants were then collected into small Eppendory tubes and stored at -70°C until used for activity studies.

(12) 0.1 M KP buffer (pH 7.4) was added to the microsomal fractions.

(13) The microsomal fractions were then vortexed to dislodge the microsomes while leaving the glycogen stuck in their places.

(14) The microsomes were each put into a clean centrifuge tube and homogenized.

(15) The microsomes were centrifuged at 37,000 rpm for 60 minutes.

(16) The supernatants were discarded and the pellets were resuspended in about 0.5 to 1 ml of 0.1 M KP buffer (pH 7.8) and then vortexed.

(17) The vortexed supernatants from step 17 were then homogenized. These are the microsomes. The microsomes were collected into small Eppendory tubes and freezed at -70°C until used for studies.

NOTE: IN THE PROCESS OF HOMOGENIZING, THE TEST TUBE CONTAIINING THE LIVER OF RAT 3 BROKE. THE LIVER OF RAT 3 WAS NOT AVAILABLE FOR USE IN THIS PROJECT.

4.4 Phase III: Protein determination in liver cytosols and liver microsomes using the Lowry method

4.4.1 Theory

The Lowry assay is a technique used for measuring protein concentration in solution. This assay is widely used and is one of the most sensitive method for determining protein concentration since it can detect protein concentration as low as 5 μg. Alkaline copper sulfate ($CuSO_4$), also known as buiret reagent, is added to a solution containing protein. Cu^{2-}, from the alkaline copper sulfate solution, coordinates with the peptide nitrogen atoms of the solution to form a purple-colored complex. Then Folin reagent, chemically known as phosphomolybdate phosphotungstate, is added to this purple-colored complex. The Folin reagent is reduced by the tyrosine and tryptophan residues from the protein resulting in the

formation of a blue-colored solution. The intensity of the blue color depends on the protein concentration in the solution.

4.4.2 Setting the Novaspec spectrophotometer for use

The absorbance on the Novaspec spectrophotometer was set at 660 nm and the reading on its screen was set to four places after the decimal. A spectrophotometer test tube filled with distilled water gave a reading of zero.

4.4.3 Standard BSA solutions

Bovine Serum Albumin (BSA) was used as a standard protein. 11.70 mg of BSA was dissolved in 11.70 ml of distilled water in a test tube to make 1 mg/ml of BSA solution. Table 2 indicates the quantities of BSA solutions used as blanks and standards for the Lowry assay.

Table 2: Quantities of BSA solutions used as blanks and standards for the Lowry assay.

Test tube #	volume of BSA solution (μl)	volume of distilled water (μl)	total volume in the test tube (μl)	BSA concentration (μg/ml)
1	none	1000	1000	0
2	none	1000	1000	0
3	50	950	1000	50
4	50	950	1000	50
5	100	900	1000	100
6	100	900	1000	100

4.4.4 Dilution of the liver cytosolic and liver microsomal samples for short term studies

1:250 dilution

For each liver cytosolic and liver microsomal sample in the short term studies, 1:250 dilution and 1:500 dilution were made.

- 1:250 dilution of a liver sample was made by micropipetting 30µl of the liver sample into a test tube containing 7.47 ml of distilled water.

1:500 dilution

- 1:500 dilution of a liver sample was made by micropipetting 30 µl of the protein sample into a test tube containing 14.97 ml of distilled water.

4.4.5 Dilution of the liver cytosolic and liver microsomal samples for long term studies

1:1000 dilution

Each liver cytosolic and liver microsomal sample for the long term studies appeared to be very concentrated in protein. Therefore 1:500 and 1:1000 dilutions were made for each liver cytosolic and liver microsomal samples. 1:500 dilution was made in the same way as for the short term studies.

- 1:1000 dilution of the liver sample was made by micropipetting 30µl of the protein sample into a test tube containing 29.97 ml of distilled water.

4.4.6 Working solution

The working solution was prepared by adding 0.5 ml of 1% copper sulfate to a beaker containing 49 ml of 2% sodium carbonate. To this solution, 0.5 ml of 2% sodium tartarate was added. If, for example, for the Lowry assay, there are a total of 38 test tubes. For each test tube, 4 ml of working solution is needed. For a total of 38 test tubes, 152 ml (38 x 4 = 152 ml) of working solution is needed. A total of 160 ml of working solution will be made, a little more than actually needed in case a test tube containing the working solution breaks. To make 160 ml of working solution, 1.60 ml of 1% copper sulfate solution is pipetted into a beaker containing 156.8 ml of 2% sodium carbonate. To this solution, 1.60 ml of 2% sodium tartarate is pipetted.

4.4.7 1 N Folin Reagent

1 N Folin Reagent was made from the available 2 N Folin Reagent by adding an equal part of Folin Reagent to an equal part of water (e.g. 10 ml of 2 N Folin Reagent in 10 ml of distilled water to make 1 N Folin Reagent).

4.4.8 Procedure

1 ml of the diluted liver sample was added into a spectrophotometer tube. To this, 4 ml of working solution was added. 4 ml of working solution was also added to the blank test tubes containing the BSA solutions (as mentioned in Table 2). The test

tubes were vortexed immediately and then left to stand in a test tube rack for strictly 10 minutes.

After 10 minutes, 0.5ml of 1 N Folin Reagent was added to each test tube. Then the test tubes was vortexed immediately and allowed to stand in the test tube rack for strictly 30 minutes. After 30 minutes, the absorbance of the solutions from each test tubes were recorded at 660 nm on the Novaspec spectrophotometer. These absorbance readings are reported in tables 8a, 8b, 9a and 9b.

4.5 Phase IV: Measuring the specific activities of GST in liver cytosols using CNDB as the substrate

4.5.1 Theory

GSH S-transferase (GST) is an enzyme which catalyzes the reduction of epoxides and dechlorination of various molecules particularly 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB). In dechlorination reaction, the chlorine in CDNB and DCNB is replaced by the -SH group of GSH. This reaction increases the water solubility of the product. The increase in the absorbance of the product is noted for calculating the total GST activity.

4.5.2 Setting the UV-VIS scanning spectrophotometer

Before starting the experiment, the UV-VIS scanning spectrophotometer's absorbance was set at 340 nm. The range, of the UV-VIS scanning spectrophotometer

was set to a value of 0 to 1. The graph generator was set at 1000 mV and its speed was set at 10 mm/minute.

4.5.3 Preparation of CDNB solution

10.1 mg of CDNB was dissolved in 1 ml ethanol in a test tube.

4.5.4 Preparation of GSH solution

31 mg of GSH was dissolved in 1 ml of distilled water in a test tube.

4.5.5 Dilution of liver cytosolic samples

1:10 dilution

- 1:10 dilution of a liver cytosol sample was made by micropipetting 50 µl of the liver cytosol into a test tube containing 450 µl distilled water.

4.5.6 Quantities of the diluted liver cytoslic samples used

15 µg of 1:10 dilution of a liver cytosol was used in this experiment. Table 3 indicates the quantities of 1:10 dilution of the liver cytosols used and the quantities of each reagent added to the standard and reactant cuvettes in this experiment.

4.5.7 Procedure

First the CDNB solution was added to both the reaction and standard cuvettes containing 0.1 M KP buffer (pH 7.8). The solution was mixed before addition of 15 µg of 1:10 dilution of liver cytosols. The solution was again mixed and then the

reactant and standard cuvettes were put in the UV-VIS scanning spectrophotometer. To the reaction cuvette, 30 µl of the GSH solution was added and the solution in the reaction cuvette was mixed by use of a toothpick. Graphs were generated and the results of this experiment are in Table 10.

Table 3: Amount of reactants added to the standard and reactant cuvettes in experiment to determine the specific activities of GST in liver cytosols using CDNB as the substrate.

Test tube #	Amount of 1:10 dilution of liver cytosol (µl) used as 15 µg♣	Amount of CDNB (µl)	Amount of 0.1M KP buffer, pH 7.8 (ml)	Total amount in cuvette (ml)
L1C	5.96	60	2.93	2.99
L2C	7.18	60	2.93	2.99
L3C	5.38	60	2.93	2.99
L5C	9.68	60	2.93	3.00
L6C	13.1	60	2.93	2.99
L7C	6.00	60	2.93	2.99
L8C	5.85	60	2.93	2.99
L9C	5.81	60	2.93	2.99
L10C	6.00	60	2.93	2.99
L11C	3.22	60	2.93	2.99
L12C	7.26	60	2.93	2.99
L13C	7.31	60	2.93	2.99
L14C	4.85	60	2.93	2.99
L15C	5.11	60	2.93	2.99
L16C	5.46	60	2.93	2.99

♣ refer to appendix A for calculations of quantities (µl) of 1:10 diluted liver cytosolic samples used as 15 µg.

4.6 Phase V: Measuring the specific activities of GST in liver cytosols using DCNB as the substrate

This phase of the experiment was carried out in almost the same way as phase IV. Differences in the procedure between this phase and phase IV are mentioned here.

4.6.1 Setting the UV-VIS scanning spectrophotometer

The UV-VIS scanning spectrophotometer was set in the same way as for phase IV. But the absorbance of the UV-VIS scanning spectrophotometer was set at 345 nm and the speed of the graph generator, connected to the UV-VIS scanning spectrophotometer, was set at 5 mm/minute.

4.6.2 Preparation of DCNB solution

9.55 mg of DCNB was dissloved in 1 ml of ethanol in a test tube.

4.6.3 Preparation of GSH solution

This solution was prepared in the same way as in phase IV.

4.6.4 Quantities of liver cytosols used

15μg of 1:10 diluted liver cytosols were also used in this experiment. The quantities of 1:10 diluted liver cytosols and the reagents used in this experiment are the same as indicated in table 3. However, 60 μl of DCNB solution (as prepared in section 4.6.2) was used instead of 60 μl of CDNB solution.

4.6.5 Procedure

First the DCNB solution was added to both the reaction and standard cuvettes containing 0.1 M KP buffer (pH 7.8). The solution was mixed and then 15 μg of 1:10 diluted liver cytosol was added to the cuvettes. The solution was again mixed.

Then the experiment was carried out in the same way as in phase IV. Graphs were generated and the results of this experiment are in table 11.

4.7 Phase VI: Measuring the cytochrome P450-dependent EROD activities in liver microsomes of rats

4.7.1 Theory

7-ethoxyresorufin and 7-pentoxyresorufin have no fluorescence. In the presence of NADPH (nicotinamide adenine dinucleotide phosphate) and oxygen, 7-ethoxyresorufin is converted into 7-hydroxyresorufin by the enzyme cytochrome P450 1A1/2 and 7-pentoxyresorufin is converted into 7-hydroxyresorufin by the enzyme cytochrome P450 2B1. The general chemical reactions are as follows:

$$\underset{\text{(7-ethoxyresorufin)}}{NADPH + O_2 + H_5C_2O\text{-resorufin}} + \text{cytochrome P450 1A1/2} \rightarrow NADP^+ + H_2O + CH_3CHO + \underset{\text{(hydroxyresorufin)}}{HO\text{-resorufin}}$$

$$\underset{\text{(7-pentoxyresorufin)}}{NADPH + O_2 + H_{11}C_5O\text{-resorufin}} + \text{cytochrome P450 2B1} \rightarrow NADP^+ + H_2O + CH_3CHO + \underset{\text{(hydroxyresorufin)}}{HO\text{-resorufin}}$$

Addition of methanol stops these reactions. The product, hydroxyresorufin, has fluorescence which is measured and used for the calculation of EROD activities and PROD activities respectively.

4.7.2 Adjusting the UV spectrofluorometer

The UV spectrofluorometer was adjusted each time it was turned on. After turning on the spectrofluorometer, the gears at each side of the spectrofluorometer were set at 5. Then the UV spectrofluorometer was allowed to warm up for at least five minutes. The excitation wavelength was set at 350 nm (slit 5) and the emission wavelength was set at 440 nm (slit 5). A clean cuvette was filled with quinone sulfate

standard solution which was allowed to come to room temperature. The cuvette was then put in the UV spectrofluorometer and a reading was obtained which was between 1.5 to 2.0. Factor was calculated to give a reading of 50. For example, if a reading of 2.0 is obtained with the quinone sulfate solution in the spectrofluorometer, then the factor is 25 (50 / 2 = 25). The factor was punched on the keyboard of the UV spectrofluorometer. Then, the fixed scale button was punched on the UV spectrofluorometer. The wavelength on the UV spectrofluorometer was changed to the assay requirement which was excitation 550 nm, slit 5 and emission 585 nm, slit 5. The cuvette containing the quinone sulfate solution was removed from the UV spectrofluorometer which was then ready for taking readings from this experiment.

4.7.3 Standard values

The graph for the standard values for this experiment was generated by using 7-hydroxyresorufin. Table 4 gives the quantities of 7-hydroxyresorufin used to generate the graph for the standard values. This experiment was done on duplicates. 7-hydroxyresorufin was added to a test tube containing buffer. The solution was mixed and then 2.5 ml of methanol was added. The resulting solution was again mixed. Finally, the solution was transferred to a cuvette for taking readings on the UV spectrofluorometer which was set at excitation wavelength 550 nm (slit 5) and emission wavelength 585 nm (slit 5). The results are reported in table 17 in Appendix B.

4.7.4 Preparation of NADPH regenerating system solution

Table 5 gives the reagents and the quantities of reagents used to make a total of 1 ml of NADPH regenerating system. Since there were a lot of liver microsomal samples, more than one ml of NADPH regenerating system solution was made. If, for example, 6 ml of NADPH regenerating system is needed. To make a total of 6 ml of NADPH regenerating system solution, all the quantities of the reagents listed in table 5, except the buffer, are multiplied by 6. The total quantities of 1 M $MgCl_2$ + isocitric dehydrogenase are subtracted with 6 ml and the result is the amount of 0.1 M KP buffer (pH 7.8) needed to make a total of 6 ml of NADPH regenerating system solution.

4.7.5 Albumin solution

The albumin solution was made by dissolving 16 mg of BSA in 1 ml of distilled water.

4.7.6 Quantities of liver microsomes used

0.1 mg of a liver microsomal sample was used. Appendix A gives the method used in calculating µl of liver microsome used as 0.1 mg.

Table 4: Quantities of reagents needed to make total of 1 ml of NADPH regenerating system solution

Reagent	Quantity
DL isocitric acid	15 mg
NADPH	4 mg
1 M $MgCl_2$	50 µl
Isocitric dehydrogenase	105 µl
0.1 M KP buffer (pH 7.8)	845 µl

Table 5: Amount of 7-hydroxyresorufin and 0.1 M KP buffer (pH 7.8) used for generating the graph for the standard values.

Test tube#	Amount of 7-hydroxyresorufin	Amount of 0.1M KP buffer, pH7.8 (ml)	Final total concentration in test tube
blank1	-	1.25	0 pmol
blank2	-	1.25	0 pmol
1	1 µl of 10 µM	1.25	10 pmol
2	1 µl of 10 µM	1.25	10 pmol
3	2 µl of 10 µM	1.25	20 pmol
4	2 µl of 10 µM	1.25	20 pmol
5	5 µl of 10 µM	1.25	50 pmol
6	5 µl of 10 µM	1.25	50 pmol
7	10 µl of 10 µM	1.24	100 pmol
8	10 µl of 10 µM	1.24	100 pmol
9	20 µl of 10 µM	1.23	200 pmol
10	20 µl of 10 µM	1.23	200 pmol
11	50 µl of 10 µM	1.20	500 pmol
12	50 µl of 10 µM	1.20	500 pmol
13	1 µl of 1 mM	1.25	1 nmol
14	1 µl of 1 mM	1.25	1 nmol
15	2 µl of 1 mM	1.25	2 nmol
16	2 µl of 1 mM	1.25	2 nmol
17	5 µl of 1 mM	1.25	5 nmol
18	5 µl of 1 mM	1.25	5 nmol

4.7.7 Procedure

This experiment was done in duplicates of two sets. Set 1 was the control and set two was the experimental. Table 6 gives the quantities of reagents added to the two sets of test tubes in this experiment.

Albumin solution was added to a test tube containing 0.1 M KP buffer (pH 7.8). The solution was mixed and then liver microsomes were added. The solution was mixed before the addition of ER. The reaction was started by adding 125 µl of NADPH regenerating system solution to the experimental test tubes. The test tubes were vortexed immediately after addition of the NADPH regenerating system

solution and put in a moving water bath (at 37 °C) for strictly ten minutes. After 10 minutes*, 2.5 mL of methanol was added to the test tubes and then, the test tubes were vortexed. The control test tubes were treated in the same way as the experimental test tubes except that instead of 125 µl of NADPH regenerating system solution, 125 µl of 0.1M KP buffer (pH 7.8) was added.

After addition of 2.5 ml of methanol and vortexing the test tubes, the control and experimental test tubes were centrifuged for 3 minutes at 2000 rpm. For each test tube, the supernatant was poured into a clean cuvette for taking readings on the UV spectrofluorometer at emission λ 550 nm, slit 5 and excitation λ 585 nm, slit 5 at three levels of the sensitivity (5-3, 5-5 and 5-10).

Table 6: Quantities of reagents added to the control and experimental test tubes in experiment to measure the cytochrome P450-dependent EROD activities.

Test tube #	Amount of 0.1 M KP buffer, pH 7.8 (µl)	Amount of albumin solution (µl)	Amount of liver microsome(µl) used as 0.1 mg	Amount of ER♣ (µl)
L1M	1004.5	100	8.00	12.5
L2M	1000.7	100	11.8	12.5
L3M	1003.2	100	9.27	12.5
L5M	1000.3	100	12.1	12.5
L6M	1000.5	100	11.9	12.5
L7M	1003.5	100	9.00	12.5
L8M	1003.6	100	8.82	12.5
L9M	1010.6	100	2.34	12.5
L10M	1010.6	100	2.34	12.5
L11M	1009.6	100	2.84	12.5
L12M	1008.2	100	4.26	12.5
L13M	1006.9	100	5.55	12.5
L14M	1008.6	100	3.87	12.5
L15M	1007.9	100	4.60	12.5
L16M	1006.1	100	6.39	12.5

♣ ER: ethoxyresorufin

Note:

• For L1M, L2M, L5M and L6M in table 6: methanol was added 11 minutes after the addition of NADPH regenerating system solution.

• For other test tubes in table 6: methanol was added 10 minutes after the addition of NADPH regenerating system solution.

4.8 Phase VII: Measuring the cytochrome P450-dependent PROD activities in rat liver microsomes

This experiment was carried out in the same way as phase VI. The only difference was that instead of ER, pentoxyresorufin (PR) was used. Table 7 gives the quantities of reagents used in this experiment.

Table 7: Quantities of reagents added to the control and experimental test tubes in experiment to measure the cytochrome P450-dependent PROD activities.

Test tube #	Amount of 0.1 M KP buffer, pH 7.8 (μl)	Amount of albumin solution (μl)	Amount of liver microsome(μl)	Amount of PR♣ (μl)
L1M	1010.8	100	8.00	6.25
L2M	1006.9	100	11.8	6.25
L3M	1009.5	100	9.27	6.25
L5M	1006.7	100	12.1	6.25
L6M	1006.9	100	11.9	6.25
L7M	1009.8	100	9.00	6.25
L8M	1009.9	100	8.82	6.25
L9M	1016.4	100	2.34	6.25
L10M	1016.4	100	2.34	6.25
L11M	1015.9	100	2.84	6.25
L12M	1014.5	100	4.26	6.25
L13M	1013.2	100	5.55	6.25
L14M	1014.9	100	3.87	6.25
L15M	1014.2	100	4.60	6.25
L16M	1012.4	100	6.39	6.25

♣ PR: pentoxyresorufin

The experiment was carried out in the same way as phase VI.

*** For all the test tubes in table 7: methanol was added 10 minutes after the addition of NADPH regenerating system solution.**

V. RESULTS

5.1 Results for phase III: Protein determination in liver cytosols and liver microsomes for short term and long term studies

Table 8a: Results of protein determination by using the Lowry method (for short term studies).

Test tube #	Mean of OD 660 nm	µg protein / ml of protein solution	mg protein / ml of protein solution
1,2	0.040	none	-
3,4	0.230	50	-
5,6	0.395	100	-
L1M250	0.287	62.5	15.6
L2M250	0.195	42.4	10.6
L5M250	0.190	41.3	10.3
L6M250	0.192	41.9	10.5
L1M500	0.157	34.2	17.1
L2M500	0.125	27.2	13.6
L5M500	0.107	23.4	11.7
L6M500	0.110	23.9	11.9
L1C250	0.397	101	25.2
L2C250	0.330	83.5	20.9
L5C250	0.285	61.9	15.5
L6C250	0.210	45.6	11.4
L1C500	0.240	52.1	26.1
L2C500	0.195	42.3	21.2
L5C500	0.172	37.5	18.8
L6C500	0.140	30.4	15.2

The values of mg protein / ml of protein solution for 1:250 dilution were used as the quantities of protein the liver samples in other phases of this project.

Refer to appendix A for explanations of calculations involved in generation of this table.

Table 8b: Additional results of protein determination by using the Lowry method (for short term studies).

Test tube #	Mean of OD 660 nm	µg protein / ml of protein solution	mg protein / ml of protein solution
1,2	0.04	none	-
3,4	0.225	50	-
5,6	0.390	100	-
L3M250	0.242	53.8	13.5
L7M250	0.250	55.6	13.9
L8M250	0.255	56.6	14.2
L3M500	0.160	35.6	17.8
L7M500	0.150	33.3	16.7
L8M500	0.170	37.8	18.9
L3C250	0.435	116	27.9
L7C250	0.390	100	25.0
L8C250	0.400	103	25.6
L3C500	0.260	57.8	28.9
L7C500	0.222	49.4	24.7
L8C500	0.240	53.3	26.7

The values of mg protein / ml of protein solution for 1:250 dilution were used as the quantities of protein in the liver samples in other phases of this project.

Refer to appendix A for explanations of calculations involved in generation of this table.

Table 9a: Results of protein determination using the Lowry method (for long term studies).

Test tube #	Mean of OD 660 nm	µg of protein / ml of protein solution	mg of protein / ml of protein solution
1,2	0.04	none	-
3,4	0.225	50	-
5,6	0.390	100	-
L9M500	0.415	106	53.2
L10M500	0.415	106	53.2
L13M500	0.202	45.0	22.5
L14M500	0.290	64.4	32.2
L9M1000	0.232	51.7	51.7
L10M1000	0.237	52.8	52.7
L13M1000	0.130	28.9	28.8
L14M1000	0 .167	37.2	37.2
L9C500	0.232	51.6	25.8
L10C500	0.225	50.0	25.0
L13C500	0.184	41.0	20.5
L14C500	0.278	61.9	30.9
L9C1000	0.250	55.6	55.6
L10C1000	0.227	50.6	50.6
L13C1000	0.180	40.0	40.0
L14C1000	0.272	60.6	60.6

The values of mg protein / ml of protein solution for 1:500 dilution were used as the quantities of protein in the liver samples in other phases of this project.

Refer to appendix A for explanations of calculations involved in generation of this table.

Table 9b: Additional results of protein determination using the Lowry method (for long term studies).

Test tube #	Mean of OD 660 nm	µg of protein / ml of protein solution	mg protein / ml of protein solution
1,2	0.035	none	-
3,4	0.231	50	-
5,6	0.395	100	-
L11M500	0.347	87.9	43.9
L12M500	0.271	58.7	29.3
L15M500	0.251	54.3	27.1
L16M500	0.181	39.1	19.5
L11M1000	0.203	44.0	44.1
L12M1000	0.161	34.8	34.7
L15M1000	0.171	36.9	36.9
L16M1000	0.120	26.0	26.1
L11C500	0.368	93.0	46.5
L12C500	0.190	41.3	20.7
L15C500	0.271	58.7	29.3
L16C500	0.253	54.9	27.4
L11C1000	0.228	49.4	49.4
L12C1000	0.120	26.0	26.1
L15C1000	0.170	36.9	36.9
L16C1000	0.160	34.8	34.8

The values of mg protein / ml of protein solution for 1:500 dilution were used as the quantities of protein in the liver samples in other phases of this project.

Refer to appendix A for explanations of calculations involved in generation of this table.

5.2 Results for phase IV: Specific activities of GST in liver cytosols using CDNB as the substrate

Table 10: Specific activities of GST in liver cytosols using CDNB as the substrate.

Sample #	DATA (μmol/min/mg protein)		
	1	2	3
L1C	1.69	1.45	1.48
L2C	2.52	2.12	2.18
L3C	1.94	1.90	2.08
For L1C to L3C(control, short term): Mean ± SD = 1.93 ± 0.34			
L5C	1.68	1.77	1.94
L6C	1.95	1.83	2.06
L7C	2.22	2.10	2.22
L8C	1.66	1.52	1.60
For L5C to L8C(experimental, short term): Mean ± SD = 1.88 ± 0.23			
L9C	1.54	1.64	1.47
L10C	2.70	1.50	1.58
L11C	1.56	1.88	1.77
L12C	1.85	1.94	1.91
For L9C to L12C (control, long term): Mean ± SD = 1.78 ± 0.34			
L13C	1.39	1.35	1.29
L14C	1.71	1.58	1.63
L15C	1.41	0.938	1.27
L16C	0.979	1.29	0.896
For L13C to L16C(experimental, long term): Mean ± SD = 1.31 ± 0.27			

Refer to appendix A for explanations of calculations involved in generation of this table.

5.3 Results for Phase V: Measuring the specific activities of GST in liver cytosols using DCNB as the substrate

Table 11: Specific activities of GST in liver cytosols using DCNB as the substrate.

Sample #	DATA μmol/min/mg protein		
	1	2	3
L1C	0.308	0.352	0.353
L2C	0.353	0.341	0.435
L3C	0.373	0.308	0.353
For L1C to L3C (control, short term): Mean ± SD = 0.353 ± 0.037			
L5C	0.353	0.294	0.255
L6C	0.314	0.353	0.314
L7C	0.365	0.392	0.382
L8C	0.235	0.212	0.314
L5C to L8C (experimental, short term): Mean ± SD = 0.315 ± 0.058			
L9C	0.235	0.247	0.247
L10C	0.364	0.588	0.518
L11C	0.441	0.294	0.388
L12C	0.517	0.482	0.544
For L9C to L12C (control, long term): Mean ± SD = 0.406 ± 0.127			
L13C	0.306	0.275	0.196
L14C	0.247	0.271	0.318
L15C	0.318	0.212	0.341
L16C	0.157	0.153	0.265
For L13C to L16C (experimental, long term): Mean ± SD = 0.254 ± 0.063			

Refer to appendix A for explanations of calculations involved in generation of this table.

5.4 Phase VI: Measuring the cytochrome P450-dependent EROD activities in liver microsomes

5.4.1 Results from the UV spectrofluorometer

Table 12a: Readings from the UV spectrofluorometer
(excitation λ 550 nm, slit 5; emission λ 585 nm, slit 5)
for test tubes containing the NADPH regenerating system solution.

Test tube #♣	Mean reading		
	5-3	5-5	5-10
L1M+	36.3	139	620
L2M+	43.0	162	729
L3M+	21.0	77.4	354
L5M+	30.6	116	522
L6M+	46.1	76.4	80.0
L7M+	36.1	134	608
L8M+	32.0	122	550
L9M+	22.1	84.5	380
L10M+	24.5	94.6	425
L11M+	22.3	84.9	382
L12M+	21.5	80.4	366
L13M+	15.3	56.6	262
L14M+	17.2	66.6	302
L15M+	30.5	116	524
L16M+	21.2	78.9	358

♣ Note: The + sign next to the test tube label indicates that the test tube contains NADPH regenerating system solution.

Table 12b: Readings from the UV spectrofluorometer
(excitation λ550 nm, slit 5; emission λ585 nm, slit 5)
for test tubes containing NO NADPH regenerating system solution.

Test tube #♣	Mean reading		
	5-3	5-5	5-10
L1M-	6.10	22.2	106
L2M-	6.80	24.6	116
L3M-	8.60	32.8	155
L5M-	6.05	22.7	107
L6M-	6.25	23.8	111
L7M-	5.45	20.5	96.1
L8M-	4.70	17.6	82.8
L9M-	5.00	19.5	90.2
L10M-	5.55	19.9	94.2
L11M-	4.10	15.1	72.2
L12M-	4.15	15.5	73.3
L13M-	5.00	18.6	88.5
L14M-	5.15	18.9	89.6
L15M-	4.50	16.7	77.7
L16M-	4.40	17.3	80.5

♣ Note: The - sign next to the test tube label indicates that the test tube contains NO NADPH regenerating system solution.

5.4.2 P450-dependent EROD activities in liver microsomes (pmol/min/mg protein)

Table 13: P450-dependent EROD activities in liver microsomes (pmol/min/mg protein).

Sample #	pmol/min/mg protein		
	5-3	5-5	5-10
L1M	137.3	141.3	143.5
L2M	164.5	165.8	171.4
L3M	62.01	59.40	61.31
Mean ± SD for L1M to L3M (control, short term)	**121.3± 53.1**	**122.2 ± 55.7**	**125.4 ± 57.2**
L5M	111.6	113.5	116.2
L6M	180.9	63.75	192.7
L7M	153.2	151.6	157.4
L8M	136.5	139.6	143.6
Mean ± SD for L5M to L8M (experimental, short term)	**145.5 ± 29.1**	**147.4 ± 29.6**	**152.5 ± 31.8**
L9M	85.51	86.66	89.27
L10M	94.75	99.60	101.7
L11M	90.75	93.18	95.16
L12M	86.51	86.60	90.20
Mean ± SD for L9M to L12M (control, long term)	**89.37 ± 4.24**	**91.49 ± 6.21**	**94.09 ± 5.72**
L13M	51.50	50.73	53.36
L14M	60.25	63.60	65.21
L15M	130.0	131.8	137.3
L16M	84.00	82.26	85.26
Mean ± SD for L13M to L16M (experimental, long term)	**81.43 ± 35.16**	**82.11 ± 35.60**	**85.27 ± 37.08**

Refer to appendix A for explanations of calculations involved in generation of this table.

5.5 Phase VI: Measuring the cytochrome P450-dependent PROD activities in liver microsomes

5.5.1 Results from the UV spectrofluorometer

Table 14a: Readings from the UV spectrofluorometer
(excitation λ 550 nm, slit 5: emission λ 585 nm, slit 5)
for test tubes containing the NADPH regenerating system solution.

Test tube #♣	Mean reading		
	5-3	5-5	5-10
L1M+	11.8	44.1	203
L2M+	13.9	53.1	239
L3M+	10.5	39.2	182
L5M+	10.5	41.6	188
L6M+	12.3	47.7	218
L7M+	20.2	79.5	198
L8M+	12.7	47.6	221
L9M+	12.7	46.6	215
L10M+	10.8	39.9	184
L11M+	11.6	44.4	201
L12M+	12.9	51.1	227
L13M+	11.8	43.6	199
L14M+	12.9	49.6	224
L15M+	15.5	63.0	285
L16M+	14.4	55.8	255

♣ Note: The + sign next to the test tube label indicates that the test tube contains NADPH regenerating system solution.

Table 14b: Readings from the UV spectrofluorometer
(excitation λ550 nm, slit 5; emission λ585 nm, slit 5)
for test tubes containing **NO** NADPH regenerating system solution.

Test tube #♣	Reading		
	5-3	5-5	5-10
L1M-	5.55	20.1	98.5
L2M-	5.75	21.7	98.4
L3M-	6.05	22.5	105
L5M-	5.55	21.9	103
L6M-	5.70	21.1	98.5
L7M-	5.30	20.9	98.5
L8M-	6.30	24.1	112
L9M-	6.70	24.6	116
L10M-	6.80	25.8	123
L11M-	5.20	19.8	93.8
L12M-	5.10	19.7	91.8
L13M-	6.00	23.5	112
L14M-	6.85	25.3	121
L15M-	5.30	20.1	95.4
L16M-	5.20	19.5	92.6

♣ Note: The - sign next to the test tube label indicates that the test tube contains **NO** NADPH regenerating system solution.

5.5.2 P450-dependent PROD activities in liver microsomes (pmol/min/mg protein)

Table 15: P450-dependent PROD activities in liver microsomes (pmol/min/mg protein).

Sample #	pmol/min/mg protein		
	5-3	5-5	5-10
L1M	31.5	32.0	32.0
L2M	41.0	41.8	43.5
L3M	22.2	22.2	23.5
Mean ± SD for L1M to L3M (control, short term)	**31.5 ± 9.3**	**32.0 ± 9.8**	**33.1± 10.0**
L5M	25.0	26.2	26.2
L6M	33.0	35.4	36.8
L7M	74.7	78.0	81.5
L8M	32.0	31.3	33.3
Mean ± SD for L5M to L8M (experimental, short term	**41.2± 22.7**	**42.7 ± 23.8**	**44.4 + 25.1**
L9M	30.1	29.4	30.7
L10M	20.1	18.8	18.9
L11M	32.2	32.8	33.2
L12M	39.2	41.7	41.8
Mean ± SD for L9M to L12M (control, long term)	**30.4 ± 7.9**	**30.7 ± 9.4**	**31.2± 9.4**
L13M	29.3	26.8	27.2
L14M	30.5	32.4	31.6
L15M	51.2	57.2	58.4
L16M	46.2	48.4	49.9
Mean ± SD for L13M to L16M (experimental, long term)	**39.3 ± 11.1**	**41.2 ± 14.1**	**41.8 ± 14.8**

Refer to appendix A for explanations of calculations involved in generation of this table.

VI. Statistical analysis of data

The t-test was performed to find out the differences between the means of two sets of samples of interest. The α level of 0.05 was chosen as the point of statistical significance.

6.1 T-tests for comparison of means of GST activities (μmol/minute/mg protien) in liver cytosols using CDNB as the substrate.

6.1.1 T-test for comparison of means of GST activities in liver cytosol between short term control rats and short term experimental rats.

Samples #	Mean ± SD
L1C to L3C	Mean ± SD = 1.93 ± 0.34
L5C to L8C	Mean ± SD = 1.88 ± 0.23

t-test value = 0.4029

α value = 0.05

critical value of Student's t distribution, $t_{0.05\,(19)} = 2.093$

t-test value < $t_{0.05\,(19)}$.

Result: **NO DIFFERENCES** between means of short term control rats and short term experimental rats.

6.1.2 T-test for comparison in GST activities between means of long term control rats and long term experimental rats.

Samples #	Mean ± SD
L9C to L12C	Mean ± SD = 1.78 ± 0.34
L13C to L16C	Mean ± SD = 1.31 ± 0.27

t-test value = 3.750

α value = 0.05

critical value of Student's t distribution, $t_{0.05\,(22)} = 2.074$

t-test value > $t_{0.05\,(22)}$.

Result: **DIFFERENCES** between means of long term control rats and long term experimental rats.

6.2 T-test for comparison of means in GST activities in (μmol/minute/mg protein) in liver cytosol using DCNB as the substrate.

6.2.1 T-test for comparison of means of GST activities in liver cytosol between short term control rats and short term experimental rats.

Samples #	Mean ± SD
L1C to L3C	Mean ± SD = 0.353 ± 0.037
L5C to L8C	Mean ± SD = 0.315 ± 0.058

t-test value = 1.713

α value = 0.05

critical value of Student's t distribution, $t_{0.05\,(19)} = 2.093$

t-test value < $t_{0.05\,(19)}$.

Result: **NO DIFFERENCES** between means of short term control rats and short term experimental rats.

6.2.2 T-test for comparison of means of GST activities in liver cytosol between long term control rats and long term experimental rats.

Samples #	Mean ± SD
L9C to L12C	Mean ± SD = 0.406± 0.127
L13C to L16C	Mean ± SD = 0.254 ± 0.063

t-test value = 3.714

α value = 0.05

critical value of Student's t distribution, $t_{0.05\,(22)} = 2.074$

t-test value > $t_{0.05\,(22)}$.

Result: **DIFFERENCES** between means of long term control rats and long term experimental rats.

6.3 T-tests for comparison of means of cytochrome P450-dependent EROD activities (pmol/min/mg protein) in liver microsomes.

6.3.1 T-test for comparison of means of cytochrome P450-dependent EROD activities between short term control rats and short term experimental rats at UV spectrofluorometer sensitivity of 5-3.

Samples #	Mean ± SD
L1M to L3M	Mean ± SD = 121.3± 53.1
L5M to L8M	Mean ± SD = 145.5 ± 29.1

t-test value = -0.7834

α value = 0.05

critical value of Student's t distribution, $t_{0.05\,(5)} = 2.571$

t-test value < $t_{0.05\,(5)}$.

Result: **NO DIFFERENCES** between means of short term short rats and short term experimental rats.

6.3.2 T-test for comparison of means of cytochrome P450-dependent EROD activities between long term control rats and long term experimental rats at UV spectrofluorometer sensitivity of 5-3.

Sample #	pmol/min/mg protein
L9M to L12M	Mean ± SD = 89.37± 4.24
L13M to L16M	Mean ± SD = 81.43± 35.16

t-test value = 0.4483

α value = 0.05

critical value of Student's t distribution, $t_{0.05\,(6)} = 2.447$

t-test value < $t_{0.05\,(6)}$.

Result: **NO DIFFERENCES** between means of long term control rats and long term experimental rats.

6.3.3 T-test for comparison of means of cytochrome P450-dependent EROD activities between short term control rats and short term experimental rats at UV spectrofluorometer sensitivity of 5-5.

Samples #	Mean ± SD
L1M to L3M	Mean ± SD = 122.2 ± 55.7
L5M to L8M	Mean ± SD = 147.4 ± 29.6

t-test value = -0.7849

α value = 0.05

critical value of Student's t distribution, $t_{0.05(5)} = 2.571$

t-test value < $t_{0.05(5)}$.

Result: **NO DIFFERENCES** between means of short term control rats and short term experimental rats.

6.3.4 T-test for comparison of means of cytochrome P450-dependent EROD activities between long term control rats and long term experimental rats at UV spectrofluorometer sensitivity of 5-5.

Samples #	Mean ± SD
L9M to L12M	Mean ± SD = 91.49 ± 6.21
L13M to L16M	Mean ± SD = 82.11 ± 35.60

t-test value = 0.5193

α value = 0.05

critical value of Student's t distribution, $t_{0.05(6)} = 2.447$

t-test value < $t_{0.05(6)}$.

Result: **NO DIFFERENCES** between means of long term control rats and long term experimental rats.

6.3.5 T-test for comparison of means of cytochrome P450-dependent EROD activities between short term control rats and short term experimental rats at UV spectrofluorometer sensitivity of 5-10.

Samples #	Mean ± SD
L1M to L3M	Mean ± SD = 125.4 ± 57.2
L5M to L8M	Mean ± SD = 152.5 ± 31.8

t-test value = -0.8108

α value = 0.05

critical value of Student's t distribution, $t_{0.05(5)} = 2.571$

t-test value < $t_{0.05(5)}$.

Result: **NO DIFFERENCES** between means of short term control rats and short term experimental rats.

6.3.6 T-test for comparison of means of cytochrome P450-dependent EROD activities between long term control rats and long term experimental rats at UV spectrofluorometer sensitivity of 5-10.

Samples #	Mean ± SD
L9M to L12M	Mean ± SD = 94.09 ± 5.72
L13M to L16M	Mean ± SD = 85.27 ± 37.08

t-test value = 0.4704

α value = 0.05

critical value of Student's t distribution, $t_{0.05(6)} = 2.447$

t-test value < $t_{0.05(6)}$.

Result: **NO DIFFERENCES** between means of long term control rats and long term experimental rats.

6.4 T-tests for comparison of means of cytochrome P450-dependent PROD activities in liver microsomes.

6.4.1 T-test for comparison of means of cytochrome P450-dependent PROD activities between short term control rats and short term experimental rats at UV spectrofluorometer sensitivity of 5-3.

Samples #	Mean ± SD
L1M to L3M	Mean ± SD = 31.5 ± 9.3
L5M to L8M	Mean ± SD = 41.2 ± 22.7

t-test value = -0.6850

α value = 0.05

critical value of Student's t distribution, $t_{0.05\,(5)} = 2.571$

t-test value < $t_{0.05\,(5)}$

Result: **NO DIFFERENCES** between means of short term control rats and short term experimental rats.

6.4.2 T-test for comparison of means of cytochrome P450-dependent PROD activities between long term control rats and long term experimental rats at UV spectrofluorometer sensitivity of 5-3.

Samples #	Mean ± SD
L9M to L12M	Mean ± SD = 30.4 ± 7.9
L13M to L16M	Mean ± SD = 39.3 ± 11.1

t-test value = -1.306

α value = 0.05

critical value of Student's t distribution, $t_{0.05\,(6)} = 2.447$

t-test value < $t_{0.05\,(6)}$.

Result: **NO DIFFERENCES** between means of long term control rats and long term experimental rats.

6.4.3 T-test for comparison of means of cytochrome P450-dependent PROD activities between short term control rats and short term experimental rats at UV spectrofluorometer sensitivity of 5-5.

Samples #	Mean ± SD
L1M to L3M	Mean ± SD = 32.0 ± 9.8
L5M to L8M	Mean ± SD = 42.7 ± 23.8

t-test value = -0.7205

α value = 0.05

critical value of Student's t distribution, $t_{0.05(5)} = 2.571$

t-test value < $t_{0.05(5)}$.

Result: **NO DIFFERENCES** between means of short term control rats and short term experimental rats.

6.4.4 T-test for comparison of means of cytochrome P450-dependent PROD activities between long term control rats and long term experimental rats at UV spectrofluorometer sensitivity of 5-5.

Samples #	Mean ± SD
L9M to L12M	Mean ± SD = 30.7 ± 9.4
L13M to L16M	Mean ± SD = 41.2 ± 14.1

t-test value = -1.239

α value = 0.05

critical value of Student's t distribution, $t_{0.05(6)} = 2.447$

t-test value < $t_{0.05(6)}$.

Result: **NO DIFFERENCES** between means of long term control rats and long term experimental rats.

6.4.5 T-test for comparison of means of cytochrome P450-dependent PROD activities between short term control rats and short term experimental rats at UV spectrofluorometer sensitivity of 5-10.

Samples #	Mean ± SD
L1M to L3M	Mean ± SD = 33.1 ± 10.0
L5M to L8M	Mean ± SD = 44.4 ± 25.1

t-test value = -0.7238

α value = 0.05

critical value of Student's t distribution, $t_{0.05(5)} = 2.571$

t-test value < $t_{0.05(5)}$.

Result: **NO DIFFERENCES** between means of short term control rats and short term experimental rats.

6.4.6 T-test for comparison of means of cytochrome P450-dependent PROD activities between long term control rats and long term experimental rats at UV spectrofluorometer sensitivity of 5-10.

Samples #	Mean ± SD
L9M to L12M	Mean ± SD = 31.2 ± 9.4
L13M to L16M	Mean ± SD = 41.8 ± 14.8

t-test value = -1.211

α value = 0.05

critical value of Student's t distribution, $t_{0.05(6)} = 2.447$

t-test value < $t_{0.05(6)}$.

Result: **NO DIFFERENCES** between means of long term control rats and long term experimental rats.

VII. Summary of results

Table 16: Summary of the short term and long term effects of curcumin on activities of glutathione S-transferase and cytochrome P450 in livers of rats.

Enzymatic activities	short term experimental rats compared with short term control rats	long term experimental rats compared with long term control rats
GST activities using:		
CDNB as the substrate	0	-
DCNB as the substrate	0	-
cytochrome P450-dependent EROD activities at UV spectrofluorometer sensitivity of:		
5-3	0	0
5-5	0	0
5-10	0	0
cytochrome P450-dependent PROD activities at UV spectroflourometer sensitivity of:		
5-3	0	0
5-5	0	0
5-10	0	0

- sign indicates significant inhibition by curcumin. 0 indicates no significant effects by curcumin.

VIII. Discussion

Curcumin significantly inhibited GST activities in liver cytosols of rats from the long term studies as compared to controls. There was no significant inhibition of GST activities in liver cytosols from the short term studies as compared to controls. These results apply to GST activities measured by using CDNB as well as DCNB as substrates.

Nojhoff *et al.* (1993) reported that 2% curcumin in AIN 76 diet, when fed to mice for three weeks, significantly increased hepatic GST activities in the small intestine but not in the liver of mice. Susan and Rao (1992) reported that curcumin, when fed to rats at a daily dose of 250 mg/kg orally for 15 days, increased hepatic GST activities by 1.8 fold compared to control rats. Oetari *et al.* (1995) reported in an vitro study that curcumin concentrations of 0.30, 1, 3, 10 amd 30 μM were potent inhibitors of GST activities in liver cytosols isolated from rats treated with phenobarbital, β-naphtoflavone or pyrazole when CDNB was used as the substrate. GST has the affinity to bind to xenobiotics or endogenous compounds such as bilirubin and heme and prevent their oxidation. In this study, inhibition of GST activities in the long term studies indicate that curcumin is a weak antioxidant since low GST activities indicate low ability of GST to bind to xenobiotics and endogenous compounds. These results suggest that rate of GST activities have no clear relation to the dose of curcumin used or the method of administration of curcumin.

Oetari *et al.* (1995) reported rapid degradation of 25 μM solution of curcumin in 50mM phosphate buffer at pH 7.4. The absorbance of the 25 μM solution of curcumin at 426 nm decreased to approximately 50% after 5 minutes and to 10% after 10 minutes. Two new absorptions appeared at 210 nm and 262 nm. The final solution was colorless which indicated that a yellow conjugated system no longer existed in the degradation products of curcumin. The instability of 25 μM curcumin solution in 50 mM phosphate buffer at pH 7.4 was completely prevented by lowering the pH of the buffer, adding 1 mM GSH, 50 μM N-acetyl N-cysteine or rat liver cytosol.

In vitro, curcumin was found to be a potent inhibitor of cytochrome P450 1A1/2 and 2B1, measured as EROD and PROD respectively (see appendix C for this experiment). Curcumin treatment in the short term as well as in the long term studies, as compared to controls, showed no significant effects on cytochrome P450 1A1/2 and 2B1 activities measured as EROD and PROD respectively.

Cytochrome P450 is often required for activation of a variety of carcinogens. Oetari *et al.* (1995) reported that curcumin was a potent inhibitor of cytochrome P450 measured as EROD activities in β-napthtoflavone induced liver microsomes and as PROD activities in phenobarbital (PB)-induced microsomes. Soudamini and Kuttan (1989) suggested that the mechanisms of action of curcumin as an inhibitor of chemical carcinogenesis involves scavenging of peroxide and superoxide species due to the antioxidant activities of curcumin. The carcinogen B[a]P requires oxidative bioactivation to B[a]P-7,8-dihydrodiol-9,10-epoxide which is the ultimate carcinogen

known to bind to DNA (4). B[a]P is bioactivated by P450 1A1/2 (5). Huang *et al.* (1992) reported that topical application of curcumin inhibits the formation of B[a]P-DNA adducts. The tumor promotion stage is generally recognized as the most important step which produces reactive oxygen species (ROS), activates protein kinase C activities, elevates mRNA and protein levels of ornithine decarboxylase and increases transcription and translation of *c-jun*-API by the stimulation of promoters including 12-O-tetradecanoyl phorbol-13-acetate (TPA). Lu *et al.* (1993) reported that curcumin inhibits protein kinase C, ornithine decarboxylase, *c-jun*-API and 8-hydroxydeoxyguanosine (8-OH-dG) formations induced by TPA. However, the mechanisms of how curcumin can inhibit activities of these compounds is still unclear (6).

IX. References

(1) Das M., Bickers D. R. and Mukhtar H. (1984) Plant phenols as in vitro inhibitors of glutathione S-transferase(s). *Biochem. Biophys. Res. Comm.* **120**(2): 427-433.

(2) Dolphin D., Avramovic O. and Poulson R., Glutathione: chemical, biochemical, and medical aspects. Wiley-Interscience Publication, New York, 2nd edition, 1989

(3) Govindarajan V.S. (1980) Tumeric: chemistry, technology and quality. *CRC Crit. Rev. Food Sci.* **12**: 199-301.

(4) Huang M. T., Wang Z. Y., Georgiadis C. A., Laskin J. D. and Conney A. H. (1992) Inhibitory effects of curcumin on tumor initiation by benzo[a]pyrene and 7,12-dimethyl-benz[a]anthracene. *Carcinogenesis.* **13**:2183-2186.

(5) Lu, Y. P., Chang R. L., Huang M. T. and Conney A. H. (1993) Inhibitory effects of curcumin on 12-O-tetradecanoylphorbol-13-acetate-induced increase in ornithine decarboxylase mRNA in mouse epidermis. *Carcinogenesis.* **14**: 293-297.

(6) Nohjoff W. A., Groen G. M. and Peters W. H. M. (1993) Induction of rat hepatic and intestinal glutathione S-transferases and glutathione by dietary naturally occurring anti-carcinogens. *Int. J. Oncol.* **3**: 1131-1139

(7) Oetari S. and Sudibyo M. (1996) Effects of curcumin on cytochrome P450 and glutathione S-transferase activities in rat liver. *Biochem. Pharmacol.* **51**: 39-45.

(8) Oshawa A., Sugiyama Y, Inayoshi M. and Kawakishi S. (1995) Antioxidative activity of tetrahydrocurcuminoids. *Biosci. Biotech. Biochem.* **59**(9): 1602-1612.

(9) Sies M., Glutathione: Biochemical properties. Wiley-Intersciece Publication, New York, 2nd edition, 1988.

(10) Soudamini K. K. and Kuttan R. (1989) Inhibition of chemical carcinogenesis by curcumin. *J. Ethnopharmacol.* **27**: 227-233.

(11)Susan M. and Rao M.N.A. (1992) Induction of glutathione S-transeferase activity by curcumin in mice. *Arzneim Drug Res.* **42**(II)(7): 962-964.

(12) Tennesen H.H., Smistad G., Agren T. and Karlsen J. (1993) Studies on curcumin and curcuminoids: XXII. Effect of curcumin on liposomal lipid peroxidation. *Int. J. Pharm.* **90**: 221-228.

(13) Tennesen H. H. and Greenhill J. V. (1992) Studies on curcumin and curcuminoids. XXII: Curcumin as a reducing agent and as a radical scavenger. *Int. J. Phar.* **87**: 79-87.

(14) Tennesen H. H. (1989a) Studies on curcumin and curcuminoid. XIII: Catalytic effects of curcumin on the peroxidation of linoleic acid by 15-lipoxygenase. *Int. J. Pharm.* **50**: 67-69.

(15) Tennesen H. H. and Karlsen J. (1985) Studies on curcumin and curcuminoids. V: Alkaline degradation of curcumin. *Z. Lebensm. Unters. Forch.* **180**: 132-134.

X. Appendix A

NOTE

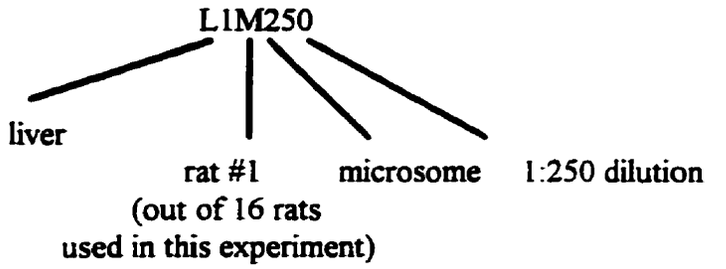
- **RAT 1 TO RAT 3 ARE CONTROLS FOR SHORT TERM STUDIES.**
- **RAT 5 TO RAT 8 ARE EXPERIMENTALS FOR SHORT TERM STUDIES.**

- **RAT 9 TO RAT 12 ARE CONTROLS FOR LONG TERM STUDIES.**
- **RAT 13 TO RAT 16 ARE EXPERIMENTALS FOR LONG TERM STUDIES.**

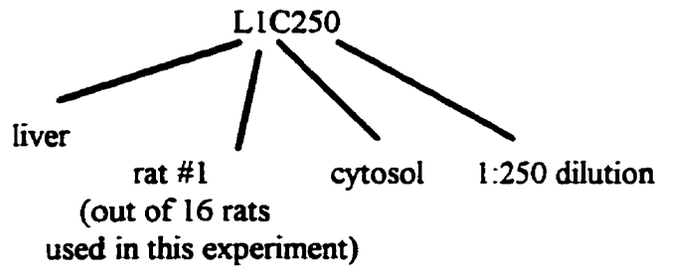
10.1 Phase III: Protein determination using the Lowry method

10.1.1 Explanation of abbreviations for the test tube # column

For L1M250:



For L1C250:



10.1.2 Calculation for μg protein/ml of protein solution

In this report, the μg protein/ml of protein solution was calculated quantitatively by comparing the readings of the mean OD 660 nm for the protein samples with the mean OD 600 nm for the BSA solutions. The calculation is demonstarted for L1M250.

For L1M250 (table 8a), the mean of OD 660 nm is 0.287. This reading is close to mean OD 660 nm reading for test tubes 3,4 which is 0.230. If 0.230 corresponds to 50μg of protein per ml of protein solution, then :

0.287 x 50μg protein/0.230 = 62.5 μg protein per ml of protein solution.

The calculations for μg of protein per ml of protein solution for other protein samples, in tables 8a to 9b, were done in the same way as demonstarted here.

10.1.3 mg of protein per ml of protein solution

mg of protein per ml of protein solution for the protein samples in tables 8a to 9b were calculated by using the formula:

(μg of protein/ml protein solution) x (dilution factor) x (1 mg/1000 μg)

The use of this formula is demonstarted for L1M250 (table 8a):

For L1M250:

- 62.5 μg protein/ml of protein solution
- dilution factor: 250

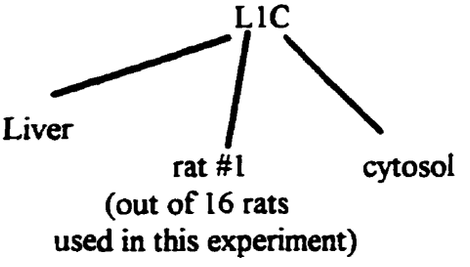
(62.5 μg protein/ml of protein solution) x (250) x (1 mg/1000 μg) =

15.6 mg protein/ 1 ml of protein solution.

10.2 Phase IV: Measuring the specific activities of GST in liver cytosols using CDNB as the substrate

10.2.1 Explanation of abbreviation used in table 3

For L1C:



10.2.2 Calculating the amount of protein needed

15μg of 1:10 dilution of the liver cytosol sample was used in this phase of the experiment. The calculation is demonstrated for L1C:

For L1C, 1:250 dilution from table 8a gives the value of 25.2 mg protein/ml of protein solution. L1C, like other liver cytosolic samples in this project, was diluted 1:10. In L1C, the mg of protein/ml of protein solution after 1:10 dilution is:

(25.2 mg of protein/ml of protein solution)/10 = 2.52.

For 15μg protein, the amount of 1:10 dilution of L1C needed is:

15/2.52 = 5.96 μl of 1:10 dilution of L1C liver cytosol sample.

10.3 Phase IV: Specific activities of GST in liver cytosols using CDNB as the substrate

10.3.1 Calculating the specific activities of GST in liver cytosols (μmol/min/mg protein)

The speed of the graph generator, connected to the UV-VIS scanning spectrophotometer, was 10 mm/minutes. So 1 unit horizontally on the graph paper is 1 minute. If speed of the graph generator, connected to the UV-VIS scanning spectrophotometer, is 5 mm/minute then 1 unit horozintally on the graph paper is 2 minutes.

The length of the best straight line (from graph), for 5 units horizontally, is

66.5 - 26.0 = 40.5 absorbance/100 units.

The graph paper is divided into 100 units. Each unit is: 40.5/100 = 0.405 abosrbance/5 minutes.

This is the absorbance value per 5 minutes. Per minute, the absorbance value is

0.405/5 = 0.081 absorbance/minute.

The extinction coefficient for CDNB is 9.6 cm mM (i.e. 1 mM = 9.6 absorbance). For 0.081 absorbance/minute,

(0.081 absorbance/minute) x (1 mM/9.6 absorbance) = 0.00844 mM/minute

Since 1 mM = 1 μmol/ml,

0.00844 mM/minute = 0.00844 μmol/ml/minute.

The total amount of solution in the reaction cuvette and standard cuvette was 3 ml (buffer + CDNB solution + 15μg of 1:10 dilution of liver cytosol). There was 15μg protein in 3 ml total solution.

15 μg protein/3 ml solution = 5 μg protein/1 ml solution.

0.00844 μmol/ml/minute = 0.00844 μmol/5 μg protein/minute.

0.00844 μmol/5 μg protein/minute = 0.00169 μmol/μg protein/minute.

Since 1 mg = 1000 μg,

(0.00169 μmol/μg protein/minute) x (1000 μg/1 mg) = 1.69 μmol/minute/mg protein.

Specifically, GST activities (in μmol/minute/mg protein) in liver cytosols using CDNB as the substrate was calculated by using the following formula:

[(absorbance/minute) / (9.6) / (5)] x 1000 = μmol/minute/mg protein.

10.4 Phase V: Specific activities of GST in liver cytosols using DCNB as the substrate

10.4.1 Calculating the specific activities of GST in liver cytosols (μmol/min/mg protein)

The speed of the graph generator, attached to the UV-VIS scanning spectrophotometer, was 5 mm/minute. This means that 1 unit horizontally on the graph paper was 2 minutes. The extinction coefficient of DCNB is 8.5 cm mM. GST activities using DCNB as the substrate was calculated in the same way as described for phase IV.

Specifically, GST activities (in μmol/minute/mg protein) in liver cytosols using DCNB as the substrate was calculated by using the following formula:

[(absorbance/minute) / (8.5) / (5)] x 1000 = μmol/minute/mg protein.

10.5 Phase VI: Measuring the cytochrome P450-dependent EROD activities in rat liver microsomes

10.5.1 Calculating quantities of liver microsomes

For this experiment, 0.1 mg of a liver microsome was used. The values of protein content for each liver sample was taken from tables 8a, 8b, 9a and 9b. The calculation for L1M is demonstrated here.

From table 8a, L1M contains 15.6 mg protein/ml. For this experiment, 0.1 mg of protein is needed.

(0.1 mg protein) x (1 ml /15.6 mg protein) = 0.00640 ml protein solution.

0.00640 ml protein solution = 6.40 µl protein solution.

(15.6 mg protein/ml protein solution) = (0.1 mg protein/6.40 µl protein solution)

In this experiment, the total amount of all reagents added (buffer + albumin solution + liver microsome + ER/PR + NADPH regenerating system solution) was 1.25 ml.

(1.25 ml) x (6.40µl/1ml) = 8.00 µl of liver microsome.

The calculations for other liver micorosome samples was done in the same way as demonstrated for L1M.

10.5.2 Calculation of P450-dependent EROD activities in rat liver microsomes (pmol/min/mg protein)

First, the mean reading obtained from the UV spectrofluorometer for the control test tubes which contained no NADPH (from table 12b) were substracted from the mean reading for the experimental test tubes (from table 12a). The values obtained were then used with graph 1 (in Appendix B) to find the pmol of 7-hydroxyresorufin. The following values lie on the regression lines in graph 1:

- For 5-3: Flourescent value of 50 on the y-axis equals 250 pmols of 7-hydroxyresorufin.
- For 5-5: Fluorescent value of 300 on the y-axis equals 400 pmols of 7-hydroxyresorufin.
- For 5-10: Fluorescent value of 650 on the y-axix equals 200 pmols of 7-hydroxyresorufin.

These values were taken for easy usage of graph 1 to find pmols of 7-hydroxyresorufin. The values of pmol of 7-hydroxyresorufin were then divided by the length of time taken for the NADPH regenerating system to react with the solutions in the test tubes. Finally, the resulting values were divided by 0.1 mg protein (since 0.1 mg of protein was used in this experiment).

The calculation is demonstrated here for L1M. For L1M+, the fluorescent measurement on the UV spectrophotometer (for 5-3) gives a mean reading of 36.3 (from table 12a). For L1M-, the fluorescent measurement on the UV spectrophotometer (for 5-3) gives a mean reading of 6.1 (from table 12b). The difference between the two readings is:

36.3 - 6.1 = 30.2 fluorescent value.

From graph 1, for 5-3: flourescent value of 50 on the y-axis equals 250 pmols of 7-hydroxyresorufin. Fluorescent reading of 30.2 equals:

(30.2) x (250 pmol 7-hydroxyresorufin/50) = 151 pmol 7-hydroxyresorufin

Methanol was added 11 minutes after the addition of NADPH regenerating system. So,

(151 pmol)/(11 minutes) = 13.7 pmol/minute.

0.1 mg of protein was used in this experiment.

(13.7 pmol/minute)/(0.1 mg protein) = 137 pmol/min/mg protein.

The cytochrome P450-dependent activity for L1M is 137 pmol/min/mg protein.

10.6 Phase VII: Measuring the cytochrome P450-dependent PROD activities in rat liver microsomes

10.6.1 Calculation of P450-dependent PROD activities in rat liver microsomes (pmol/min/mg protein)

This was calculated in the same way as for phase VI.

XI. Appendix B

11.1 Standard values for phases VI and VII of this study

Table 17: Results for the standard values
(excitation λ 550 nm, slit 5; emission λ 585 nm, slit 5)

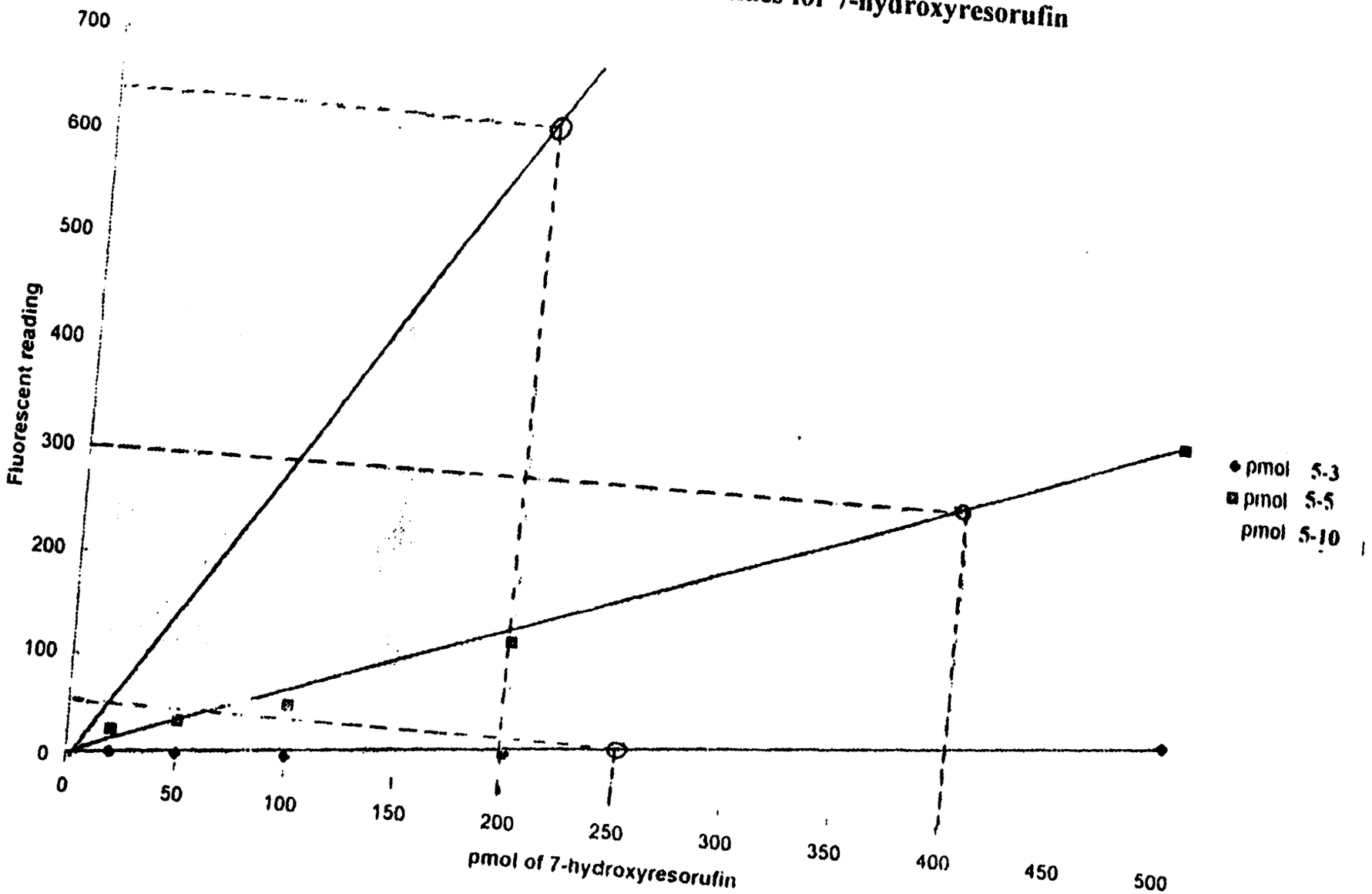
Test tube #	Total final concentration of test tube	Mean ± SD		
		5-3	5-5	5-10
blank	0pmol	0.60	0.70	2.95 ± 0.35
1	10 pmol	7.80 ± 0.70	29.4 ± 3.2	130.5 ± 14.9
2	20 pmol	7.80 ± 0.70	29.4 ± 3.2	130.5 ± 14.9
3	50 pmol	11.7± 0.6	43.2 ± 1.2	195.6 ± 6.2
4	100 pmol	17.9 ± 1.6	66.7 ± 6.9	298.3 ± 35.3
5	200 pmol	39.0 ± 0.5	145.8 ± 1.9	655.95 ± 7.8
6	500 pmol	99.8 ± 0.4	384.9 ± 1.7	-
7	1 nmol	298.9 ± 4.9	-	-
8	2 nmol	444.8 ± 24.3	-	-
9	5 nmol	797.9 ± 3.3	-	-

Graph 1 was generated by subtracting the mean values of the test tubes (1 to 9) with the mean values of the blank. Test tube 1 has a mean of 7.80, The blank has a mean of 0.60.

$$7.80 - 0.60 = 7.20$$

These values were plotted against final concentration of the test tube.

Graph 1: Standard values for 7-hydroxyresorufin



XII. Appendix C

12.1 Measuring the P450-dependent EROD activities in vitro in rat liver microsomes

12.1.1 Standard values

This was done in the same way as for phase VI (see table 5 for quantities of 7-hydroxyresorufin used to generate the standard curve).

12.1.2 Procedure

The experiment was done in duplicate using L1M in the same way as phase VI. However, 0.2 mg/ml of microsomes from L1M was used. Curcumin solutions were also used in this experiment.

12.1.2.1 Liver microsomes

Table 8a indicates that L1M contains 15.620 mg protein/ml of protein solution. For this experiment, 0.2 mg of protein is needed.

(0.2 mg protein) x (1 ml / 15.6 mg protein) = 0.0128 ml protein solution.

0.0128 ml protein solution = 12.8 µl protein solution.

(15.6 mg protein / ml protein solution) = (0.2 mg protein / 12.8 µl protein solution)

In this experiment, the total amount of all reagents added (buffer + albumin solution + liver microsome + ER/PR + NADPH regerating system) was 1.25 ml.

(1.25 ml) x (12.80 µl / 1 ml) = 16 µl of liver microsome.

12.1.2.2 Preparation of curcumin solutions

100 mM curcumin solution

36.8 mg of curcumin was dissolved in 1 ml of 0.1 M NaOH. To this solution, one drop of 5 M NaOH was added when required for complete dissolution of curcumin.

1:10 dilution of 100 mM curcumin solution

This was made by pipetting 1 ml of 100 mM curcumin solution (prepared above) in 18 ml of distilled water. Table 18 gives the quantities of reagents added to the standard and reaction test tubes.

Table 18: Quantities of reagents added to the control and experimental test tubes in experiment to measure the in vitro cytochrome P450-dependent EROD activities

Test tube #	Amount of 0.1 M KP buffer. pH 7.8 (µl)	Amount of albumin solution (µl)	Amount of liver microsome (µl)	Amount of curcumin solution (µl)	Amount of ER (µl)
blank	996.5	100	16	0	12.5
1	994.0	100	16	2.5 of 1:10 dilution	12.5
2	991.5	100	16	5 of 1:10 dilution	12.5
3	986.5	100	16	10 of 1:10 dilution	12.5
4	994.0	100	16	2.5	12.5
5	991.5	100	16	5	12.5

12.1.3 Results

Table 19: Cytochrome P450-dependent EROD activities in vitro in rat liver microsomes (pmol/min/mg protein).

Test tube #	pmol/min/mg protein		
	5-3	5-5	5-10
blank	128.7	407.9	145.9
1	97.39	364.1	1642
2	79.95	311.6	1379
3	41.20	158.6	712.8
4	15.62	59.54	264.9
5	0.3875	5.680	22.47

Calculations involved in generation of this table were done in the same way as explained in section 10.5.2 in Appendix A.

12.2 Measuring the P450-dependent PROD activities in vitro in rat liver microsomes

This experiment was carried out in duplicates and in the same way as in section 12.1 in this appendix. Table 20 gives the quantities of reagents used in this experiment.

Table 20: Quantities of reagents added to the control and exprimental test tubes in experiment to measure the P450-dependent PROD activity in vitro in rat liver microsomes.

Test tube #	Amount of 01.M KP buffer, pH 7.8 (µl)	Amount of albumin solution (µl)	Amount of liver microsome (µl)	Amount of curcumin solution (µl)	Amount of PR (µl)
blank	1002.7	100	16	0	6.25
1	1000.2	100	16	2.5 of 1:10 dilution	6.25
2	997.7	100	16	5 of 1:10 dilution	6.25
3	992.7	100	16	10 of 1:10 dilution	6.25
4	1000.2	100	16	2.5	6.25
5	997.7	100	16	5	6.25
6	992.7	100	16	10	6.25
7	1002.7	100	16	0	6.25

12.2.1 Results

Table 21: P450-dependent PROD activities in vitro in rat liver microsomes (pmol/min/mg protein).

Test tube#	pmol/min/mg protein 5-3	5-5	5-10
blank	27.9	27.6	27.2
1	22.4	24.5	24.1
2	21.7	23.1	21.8
3	19.2	18.8	18.7
4	8.65	8.01	7.96
5	13.0	13.7	13.9

Calculations involved in generation of this table were done in the same way as explained in section 10.5.2 in Appendix A.