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APPENDIX A

Nutritional composition of mice feed (C.P. Ltd, Thailand)

Moisture	(Max)	12%
Crude protein	(Min)	24%
Fat	(Min)	4.5%
Fiber	(Max)	5%
Metaboliable energy (swine) Kcal/kg		3,040
Calcium		1.4%
Phosphorus (available)		0.9%
Sodium		0.20%
Potassium		1.17%
Magnesium		0.23%
Manganese	p.p.m.	171
Copper	p.p.m.	22
Zinc	p.p.m.	100
Iron	p.p.m.	180
Cobalt	p.p.m.	1.82
Potassium Iodide	p.p.m.	1
Selenium	p.p.m.	0.1

Vitamins

A	i.u./kg	20,000
D	i.u./kg	4,000
E	mg/kg	100
K	mg/kg	5
B 1	mg/kg	20
B 2	mg/kg	20
B 6	mg/kg	20
B 12	mg/kg	0.036
Niacin	mg/kg	100
Folic acid	mg/kg	6
Biotin	mg/kg	0.4
Pantothenic acid	mg/kg	60
Choline choride	mg/kg	1,500

APPENDIX B

Controlled environmental condition of animal room (at NIH, Thailand)

Temperature	23 ± 1 °C
Relative humidity	40-70%
Light/dark cycle	12 hours/12 hours
Cage	Shoebox cage, sterilized before use and changed at least 2 times/week
Bedding	Soft-wood bedding, sterilized before use and changed at least 2 times/week

APPENDIX C

Determination of hematocrit (Shirley, 1982)

1. Materials
    1) Hematocrit capillary tube
    2) Hematocrit centrifuge

2. Procedure

The hematocrit of a blood sample is determined by filling a tube of uniform bore with blood, centrifuging the blood sample to spin down the cells, and then calculating the percentage of the total blood column that is comprised of red cells.

A capillary tube coated with heparin is used for a hematocrit tube. Tip an end the capillary tube into the blood sample in a microtip and hold the tube until it is almost filled with blood. Plug the bottom of the tube by sticking it in a small piece of clay. The bottom of the tube must be tightly sealed or the blood will be lost during centrifugation.

Place the tube in one of the numbered slots in the head of a clinical centrifuge. The plugged end of the tube must lie against the rubber cushion at the edge of the centrifuge head. Since the centrifuge head must be balanced if the centrifuge is to run smoothly, place your tube in a slot in the head directly opposite a slot containing another tube. Note the number of the slot in which you placed your tube so that the tube can be identified later. Centrifuge the blood for 10 minutes, remove the tube from the centrifuge, and determine the hematocrit . Then divide the height of the red cell column by the height of the total blood column to determine the percentage of the blood volume that consists of red blood cells. Record the hematocrit of the blood sample. The average hematocrit of Wistar rats is 42.5-49.4 % (Semler et al., 1992).

Counting the leukocytes (Shirley, 1982)

1. Materials
   1.1 Instruments
      1) Hemocytometer
      2) White cell diluting pipette
      3) Hematocrit capillary tube
      4) Light microscope
   1.2 Reagents
      1) Distilled water
      2) 3% Glacial acetic acid

2. Procedure

Examine the hemocytometer under the low power objective of a light microscope. The slide has lines etched on its surface in a pattern such as that shown in figure 23. The leukocytes that fall in four areas in this region of large squares will be counted. (Each of the four areas is marked with an "W" in figure 23). Before placing a diluted blood sample on the slide, observe the slide under the microscope until the areas in which the blood cells are to be counted can be easily recognized.

Place the tip of the white cell diluting pipette in the drop of blood and, by gently applying suction at the mouthpiece, pull a column of blood up to the 0.5 mark on the pipette. The column of blood must come exactly to that line and there must be no air spaces in the column. If blood is pulled past the mark, carefully and quickly blot the tip of the pipette with a small piece of absorbent cotton to withdraw the excess blood. Pull 3% glacial acetic acid into the pipette to the line marker 11. Use very little suction, since the fluid will fill the pipette quite rapidly. Hold thumb and middle finger over the ends of the pipette and shake the pipette back and forth in a "figure 8" movement to mix the contents. Then discard that fluid that is contained in

the capillary portion of the pipette (1-3 drops) because that fluid would not have been thoroughly mixed with the blood.

Place the cover lip on the hemocytometer so that it covers the center of the slide. Place the tip of the pipette at the edge of the coverslip (figure 24) and allow enough diluted blood to run under the coverslip to cover the grid without spilling into the moat.

When counting the cells, it is important that each cell in the area designated "W" be counted only once. Those cells that fall on a line are counted if they are on the upper or left boundary of any square in which cells are counted, but those on the other two margins of the square are not counted.

Multiply the total number of leukocytes counted by a factor of 50 to determine the number of leukocytes per cubic millimeter of blood. A count of 5-8.96 ($x10^3$) cells/mm^3 is about average of Wistar rats.

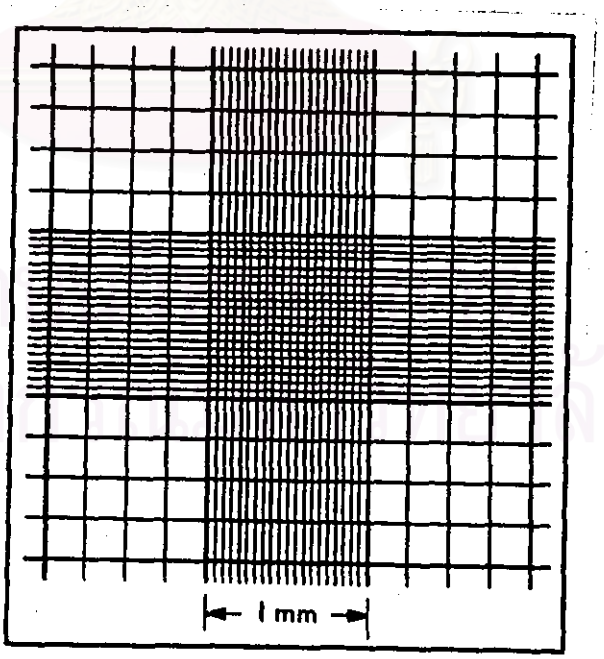


Figure 23. Rulings on hemocytometer counting chamber;
W - Areas in which leukocytes are counted.

3. Care of the glassware used in blood cell counting.

A hemocytometer counting chamber, a thick coverslip used with the chamber, and a cell diluting pipette are used for counting blood cells (figure 24). These glassware must be cleaned before use with distilled water and polished with lens paper.

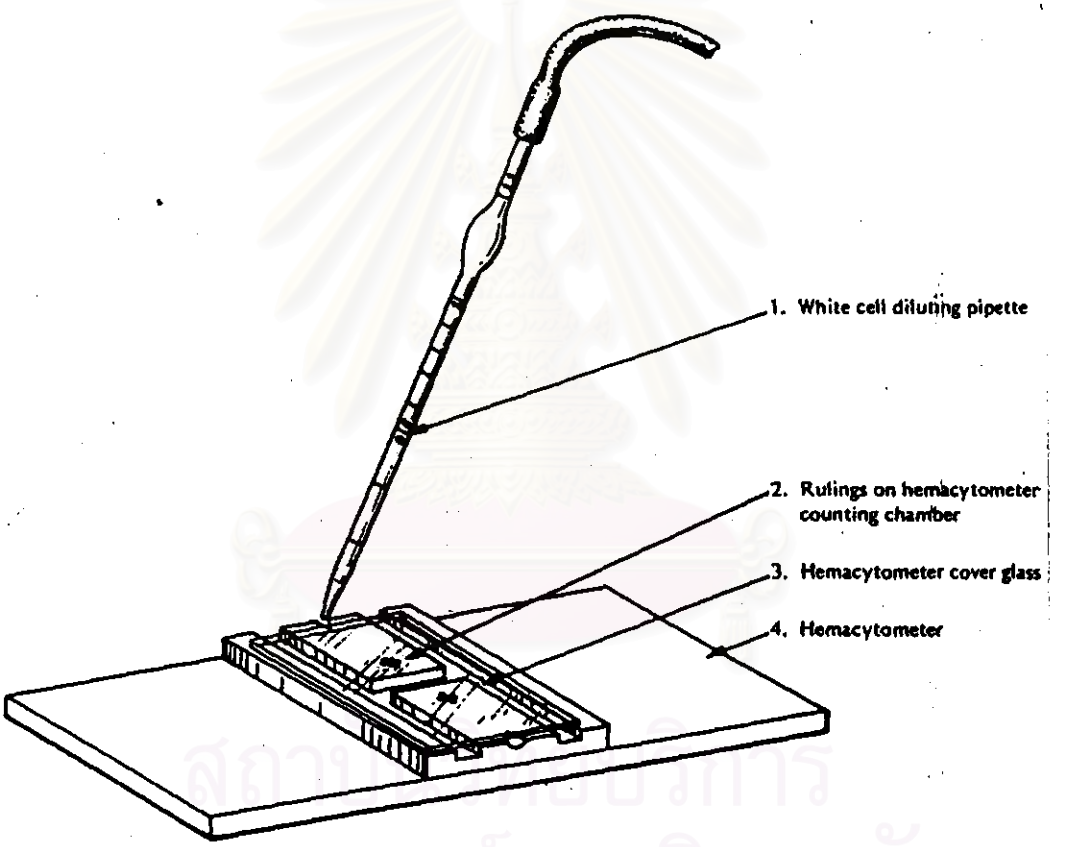


Figure 24. Technique for applying diluted blood to hemocytometer.

Preparation of a stained blood smear (Shirley, 1982)

1. Materials
   1.1 Instruments
      1) Glass slides
      2) Staining dish
   1.2 Reagents
      1) Quik stain

2. Procedure

Clean and dry two slides. Place a drop of blood collected near one end of one of the slides. With that slide in a horizontal position, place the other slide at a 45° angle to it. The angles slide should touch the edge of the drop of blood on the first slide (Figure 25) In a quick motion, move the second slide across the first, dragging (not pushing) the drop of blood across the slide in a thin, uniform layer. Allow the blood smear to dry thoroughly.

Holding the blood slide by one end, lower it into a staining dish containing. Quik-Stain (a special preparation of Wright's stain) and leave it in the stain for 5 seconds. Transfer the slide to a second dish containing the distilled water and let it remain in the water for 5-10 seconds. Rinse the slide quickly with tap water by allowing the stream of water to strike the edge of the slide and flow across the blood smear; the water should not strike the blood smear directly. Allow the slide to completely dry and keep in a slide box for further differential count.

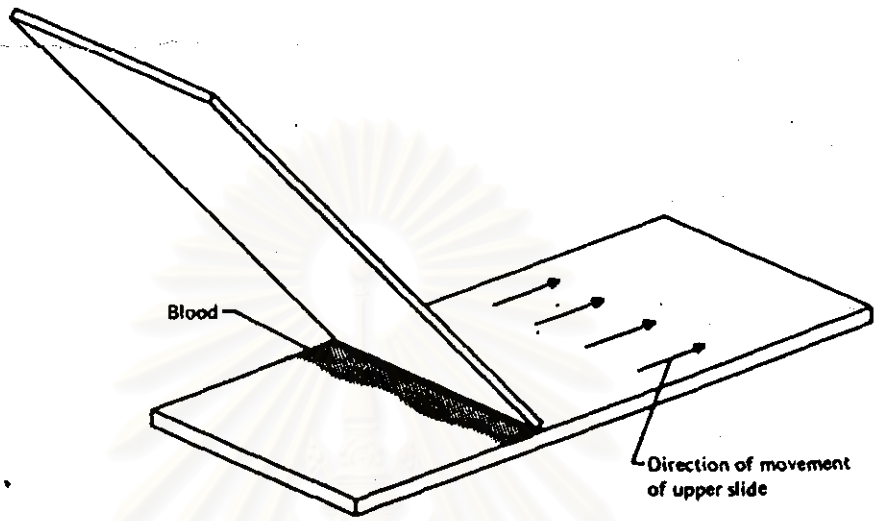


Figure 25. Preparation of a blood smear.

Differential count of leukocytes (Shirley, 1982)

The differential count of leukocytes on the stained blood smear can be performed under a light microscope using 40x and an oil immersion lens. The high power (40x) len is used for counting the cells, whereas the oil immersion lens is used for detecting the cell shape. For the oil immersion lens, place a drop of immersion oil on a region of the slide where the blood film is thin (on the end of the slide opposite that to which the drop of blood was applied). Count 100 leukocytes and categorize them according to type. While performing the differential count, move the slide in such a way that the cells are still not counted in any area on the slide more than once. After the cell count is finished, compare the percentage of the leukocytes in the blood smear with the average numbers of the various types of leukocytes seen in differential counts of normal blood samples.

Identify as many types of blood cells (figure 26) on the slide. The cells that are most numerous are erythrocytes. They are biconcave disks whereas the leukocytes are spherical. Also, erythrocytes lack nuclei, but nuclei are present in leukocytes. Since the nuclei in leukocytes are not all of the same size and shape, nuclear characteristics are useful in distinguishing the different types of leukocytes from one another.

Identify the different types of leukocytes, and the thrombocytes in the stained blood smear. Use the following description of leukocyte characteristics and the diagrams of blood cells in figure 26 as aids in the identification of the cells on the slide.

The average differential count of Wistar rats (Semler, 1992) are as follows:

Neutrophils: lobed nuclei, faintly purple granules in cytoplasm, comprise approximately 9-34 % of total number of leukocytes.

Eosinophils: lobed nuclei, red granules in cytoplasm, 0-2.5 % of total.

Basophils: bilobed nuclei, coarse blue granules in cytoplasm, 0-1.5 % of total.

Lymphocytes: large nuclei that almost fill cells, no apparent granules in cytoplasm, 65-84.5 % of total.

Monocytes: large nuclei that may appear horseshoe-shaped, nuclei not so large in relation to size of cells as nuclei of lymphocytes, 0-5 % of total.

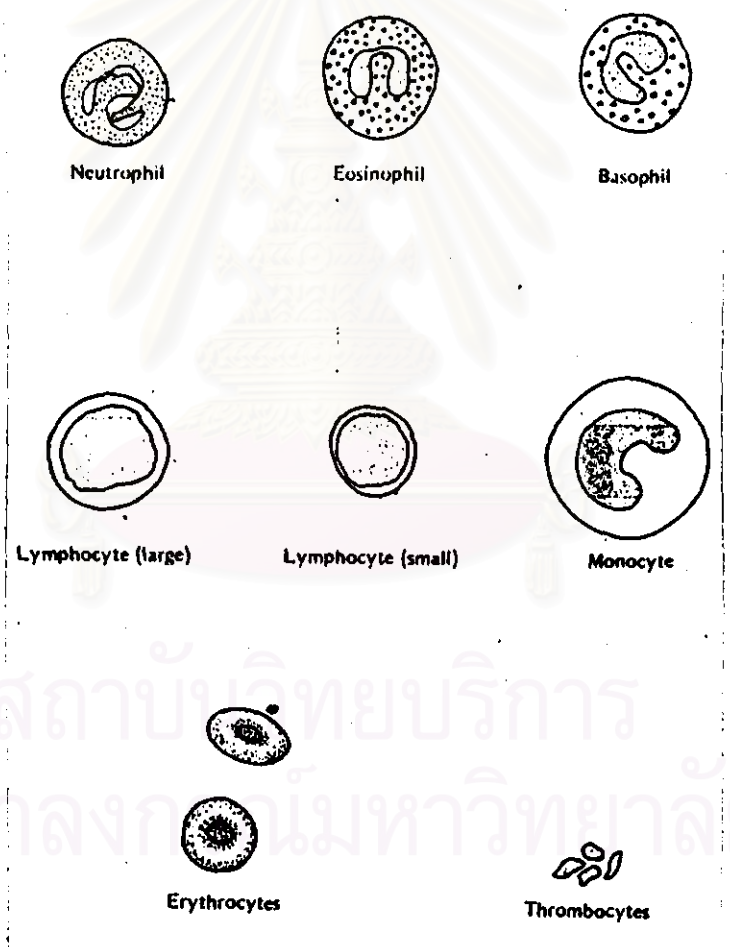


Figure 26. Blood cells.

APPENDIX D

Determination of plasma hemoglobin (Fairbank and Klee, 1994)

1. Principle

Virtually all of the hemoglobin in blood is contained within the erythrocytes. A minute quantity of hemoglobin is normally released into plasma by destruction of erythrocytes, and this is promptly bound by haptoglobin. The haptoglobin-hemoglobin complex is rapidly removed by parenchymal cells of the liver. Thus, the normal plasma hemoglobin concentration is close to zero.

In this assay, the concentration of plasma hemoglobin is measure in diluted plasma at 415, 450, and 700 nm, where A_{415} is the Soret band absorption maximum for hymoglobin (oxyhemoglobin, deoxyhemoglobin, and methemoglobin), A_{450} is the absorption maximum for bilirubin, and A_{700} is a correction for turbidity of the specimen.

2. Specimen

Measurement of plasma hemoglobin requires 0.1 ml of plasma. Heparin is a satisfactory anticoagulants when collecting blood. The plasma is separated form erythrocytes as soon as possible and the plasma recentrifuged and reseparated to remove residual erythrocytes.

3. Materials

3.1 Instruments

1) Spectrophotometer

2) Polystyrene cuvets, 1-cm light path

3.2 Reagents

1) Sodium carbonate (Na_2CO_3) stock solution. Dissolve 1 g Na_2CO_3 in 100 ml of distilled water. The solution remains stable at room temperature up to 1 year.

2) Na_2CO_3 working solution, 10 mg/100 ml. Dilute 1 ml stock solution to 100 ml with distilled water. The solution remains stable at room temperature up to 1 year.

4. Procedure

4.1 After centrifuging the sample, remove plasma and recentrifuge at 2,000g for 10 minutes to remove any residual erythrocytes.

4.2 Pipet 1.0 ml working sodium bicarbonate solution into cuvets.

4.3 Add 0.1 ml of a plasma sample into test cuvet and mix.

4.4 Measure absorbance at 415, 450 and 700 nm

5. Calculations

$$C_H = 154.7\ A_{415} - 130.7\ A4_{50} - 123.9\ A_{700}$$

where C_H = total hemoglobin concentration expressed as mg/dl

A_{415} = absorbance at 415 nm

A_{450} = absorbance at 450 nm

A_{700} = absorbance at 700 nm

6. Reference interval

Total hemoglobin concentration 0-0.1 g/L

APPENDIX E

Determination of glutamate pyruvate transaminase (GPT) activity

(Clinical Diagnostics, 1996)

1. Principle

GPT enzyme catalyze transaminase reaction of alanine and α-keto-glutarate. The products, pyruvate and glutamate, then form color complex with dinitrophenylhydrazine in NaOH solution. This complex can be measured by a spectrophotometer at 520 nm.

2. Specimen

Heparinized plasma samples are obtained by centrifugation of the blood sample collected by jugular vein cannulation as previously mentioned. These samples are kept in ice-cold temperature and are analyzed as soon as possible (within 24 hours).

3. Materials

3.1 Instruments

1) Temperature controlled water bath
2) Spectrophotometer
3) Autopipet (0.5 ml, 0.1 ml, 5 ml)

3.2 Reagents (Clinag® kit)

1) GPT substrate
2) Phenylhydrazine
3) 0.4N NaOH
4) Pyruvate standard solution

4. Procedure

4.1 Set up the a spectrophotometer to the wavelength 520 nm with distilled water as reaction blank and warm the water bath.

4.2 Pipet 0.5 ml of GPT substrate into a test tube and then place the tube in the 37°C water bath for 5 minutes. Add 0.1 ml of the plasma sample into the tube, mix rapidly and incubate in the 37°C water bath for 15 minutes exactly. Add 0.5 ml of phenylhydrazine, mix, and then place the tube in the room temperature condition for exact 20 minutes. Add 5 ml of 0.4 N NaOH into the tube, mix, wait for 5 minutes at room temperature, and next transfer to a 3-ml polystyrene cuvet with 10-mm path length. Insert the cuvet in the spectrophotometer. Read the absorbance at 520 nm.

5. Calibration curve

The calibration curve can be obtained by using standard pyruvate solution and substrate in place of the plasma sample as shown in table 24.

Table 20. Compositions of the reagents to set up the calibration curve of GPT.

Tube No.	Standard pyruvate (ml)	Substrate (ml)	Distilled water (ml)	GPT activity (SF Units/ml)
1	0	0.50	0.1	0
2	0.05	0.45	0.1	25
3	0.10	0.40	0.1	50
4	0.15	0.35	0.1	83
5	0.20	0.30	0.1	126
6	0.25	0.25	0.1	-

APPENDIX F

Table 21. GPT activity in the plasma of rats receiving an oral dose of methomyl (3-7 mg/kg) at various time points after dosing* (mean±SD; n = 4-6).

Days after dosing	Groups			
	Control	Methomyl 3 mg/kg	Methomyl 5 mg/kg	Methomyl 7 mg/kg
Day 5	21.2±1.2	20.0±1.3	19.4±2.5	18.5±2.3
Day 7	20.6±2.0	19.2±4.4	20.5±5.2	17.4±3.0

* The determination of GPT activity did not performed on the day 1 and 3 after dosing.

Table 22. GPT activity in the plasma of rats receiving 5 repeated doses of 5 mg/kg/day of methomyl at various time points after dosing (mean±SD; n = 6).

Days after dosing	Groups	
	Control	Methomyl 5 mg/kg/day (for 5 days)
Day 1	22.0±3.1	22.9±2.0
Day 3	22.5±2.6	22.5±3.4
Day 5	23.5±2.8	24.4±2.5
Day 7	22.2±2.5	21.8±3.9

APPENDIX G

Table 23. Organ weight of rats fed an acute dose (3-7 mg/kg) of methomyl at various time points after dosing (mean±S.E.; n = 6).

Organ	Day(s) after dosing	Organ weight (g)			
		Control	Methomyl 3 mg/kg	Methomyl 5 mg/kg	Methomyl 7 mg/kg
Liver	1	8.4±0.4	6.9±0.3*	6.6±0.2*	7.2±0.5*
	3	8.2±0.3	7.3±0.4*	7.7±0.3*	7.4±0.3*
	5	9.6±0.1	8.5±0.4*	8.2±0.5*	7.1±0.2*
	7	9.4±0.3	9.3±0.2	9.3±0.6	7.9±0.3*
Spleen	1	0.8±0.0	0.8±0.1	0.8±0.0	0.8±0.0
	3	1.0±0.1	0.8±0.1	1.0±0.1	0.9±0.1
	5	0.9±0.1	0.9±0.1	1.0±0.1	0.9±0.1
	7	1.0±0.1	1.1±0.1	0.9±0.1	0.9±0.0
Heart	1	0.7±0.0	0.7±0.0	0.8±0.0	0.7±0.0
	3	0.7±0.0	0.8±0.0	0.8±0.0	0.7±0.0
	5	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0
	7	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0
Kidney	1	1.5±0.1	1.5±0.1	1.6±0.0	1.5±0.1
	3	1.6±0.0	1.7±0.1	1.6±0.0	1.6±0.1
	5	1.6±0.0	1.7±0.1	1.6±0.1	1.5±0.0
	7	1.7±0.1	1.8±0.0	1.8±0.1	1.7±0.1

* Significant decrease in the relative weight when compared with the controls ($p<0.05$).
(Two-way ANOVA was used coupled with Duncan's multiple range test for statistical analysis.)

Table 24. Relative organ weight of rats fed an acute dose (3-7 mg/kg) of methomyl at various time points after dosing (mean±S.E.; n = 6).

Organs	Day(s) after dosing	Relative organ weight (% of total body weight)			
		Control	Methomyl 3 mg/kg	Methomyl 5 mg/kg	Methomyl 7 mg/kg
Liver	1	4.81±0.11	4.10±0.10*	3.90±0.20*	4.20±0.30*
	3	4.35±0.09	3.92±0.19*	4.01±0.05*	4.01±0.09*
	5	4.67±0.09	4.20±0.10*	3.98±0.15*	3.65±0.08*
	7	4.39±0.06	4.32±0.05	4.43±0.33	3.77±0.08*
Spleen	1	0.45±0.02	0.45±0.03	0.45±0.01	0.48±0.02
	3	0.50±0.03	0.45±0.04	0.50±0.03	0.50±0.03
	5	0.45±0.03	0.44±0.03	0.47±0.04	0.47±0.04
	7	0.47±0.03	0.51±0.03	0.42±0.03	0.45±0.01
Heart	1	0.41±0.02	0.39±0.02	0.44±0.02	0.43±0.02
	3	0.39±0.02	0.42±0.01	0.43±0.02	0.39±0.01
	5	0.40±0.01	0.40±0.01	0.38±0.02	0.39±0.01
	7	0.39±0.01	0.36±0.01	0.40±0.02	0.36±0.01
Kidney	1	0.87±0.02	0.87±0.03	0.91±0.03	0.87±0.02
	3	0.84±0.02	0.90±0.05	0.85±0.02	0.84±0.03
	5	0.79±0.01	0.82±0.02	0.76±0.02	0.75±0.01
	7	0.77±0.03	0.81±0.02	0.84±0.04	0.79±0.02

* Significant decrease in the relative weight when compared with the controls ($p<0.05$).
(Two-way ANOVA was used coupled with Duncan's multiple range test for statistical analysis.)

Table 25. Organ weight of rats fed 5 repeated doses of 5 mg/kg/day of methomyl at various time points after dosing (mean±S.E.; n = 6).

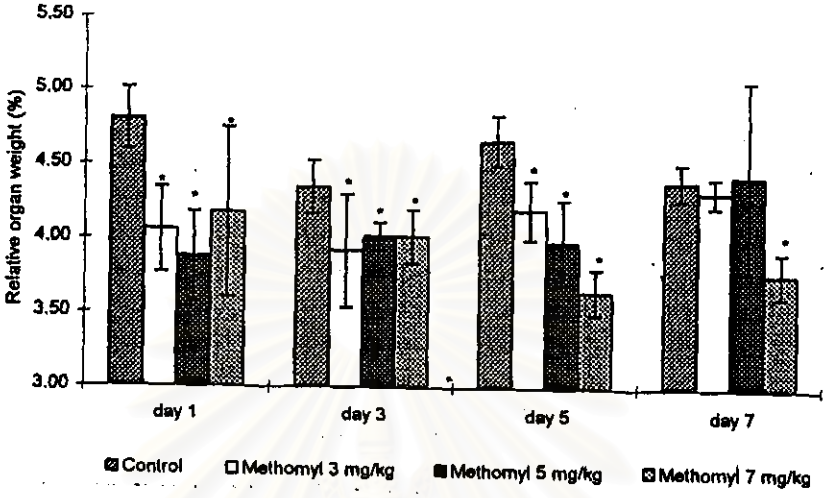
Organs	Day(s) after dosing	Organ weight (g)	
		Control	Methomyl 5 mg/kg/day (for 5 days)
Liver	1	8.7±0.2	8.6±0.5
	3	9.3±0.3	9.2±0.3
	5	10.2±0.4	10.3±0.5
	7	10.6±0.3	10.4±0.3
Spleen	1	0.9±0.1	0.9±0.1
	3	0.9±0.1	1.0±0.0
	5	1.0±0.0	0.9±0.1
	7	0.9±0.1	1.0±0.1
Heart	1	0.8±0.0	0.7±0.1
	3	0.8±0.0	0.8±0.0
	5	0.8±0.0	0.8±0.0
	7	0.9±0.0	0.9±0.0
Kidney	1	1.7±0.1	1.7±0.1
	3	1.8±0.1	1.8±0.1
	5	1.8±0.0	1.9±0.1
	7	1.9±0.1	1.8±0.1

(Two-way ANOVA was used coupled with Duncan's multiple range test for statistical analysis.)

Table 26. Relative organ weight of rats fed 5 repeated doses of 5 mg/kg of methomyl at various time points after dosing (mean±S.E.; n = 6).

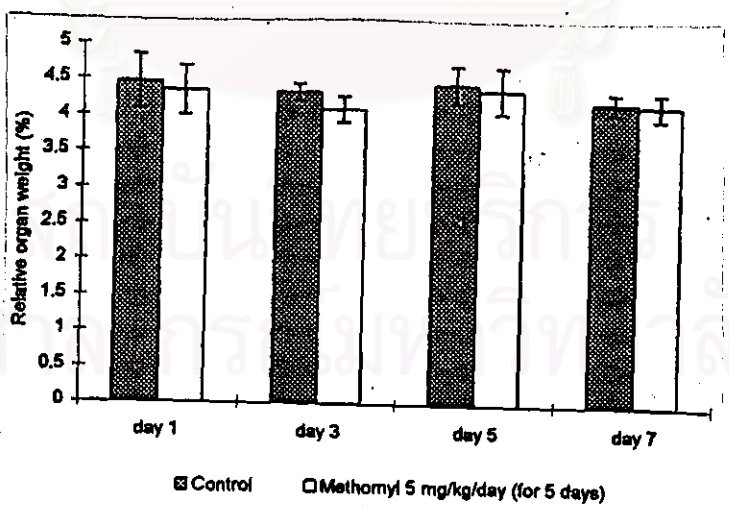
Organs	Day(s) after dosing	Relative organ weight (% of total body weight)	
		Control	Methomyl 5 mg/kg/day (for 5 days)
Liver	1	4.47±0.19	4.35±0.17
	3	4.34±0.06	4.11±0.09
	5	4.46±0.13	4.38±0.16
	7	4.22±0.07	4.19±0.09
Spleen	1	0.46±0.04	0.44±0.02
	3	0.43±0.04	0.45±0.02
	5	0.43±0.01	0.40±0.03
	7	0.37±0.02	0.41±0.02
Heart	1	0.39±0.02	0.38±0.01
	3	0.37±0.02	0.37±0.02
	5	0.35±0.01	0.35±0.02
	7	0.36±0.01	0.36±0.01
Kidney	1	0.86±0.04	0.85±0.02
	3	0.85±0.03	0.80±0.02
	5	0.77±0.01	0.79±0.02
	7	0.76±0.04	0.73±0.03

(Two-way ANOVA was used coupled with Duncan's multiple range test for statistical analysis.)



* Significant decrease in relative organ weight when compared with the controls ($p<0.05$)
(Two-way ANOVA was used coupled with Duncan's multiple range test for statistical analysis.)

Figure 27. Relative weight of liver of rats treated with an oral dose of methomyl (3-7 mg/kg) at vaious time points after dosing (mean±1.96S.E.).



* Significant decrease in relative organ weight when compared with the controls ($p<0.05$)
(Two-way ANOVA was used coupled with Duncan's multiple range test for statistical analysis.)

Figure 28. Relative weight of liver in rats treated with 5 mg/kg of methomyl for 5 days at vaious time points after last dosing (mean±1.96S.E.).

BIOGRAPHY

Mrs. Ornrat Lohitnavy was born on May 15, 1972 in Rayong Province, Thailand. She received her Bachelor's degree of Sciences in Pharmacy in 1991 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. During 1991-1993, she worked at the Office of Public Health, Chonburi Province, Thailand and thereafter she continued studying at the Faculty of Pharmaceutical Sciences for the Master's degree of Sciences in Pharmacology. After the completion of her M.S. study, she is a member at the Department of Pharmacy Practice, the Faculty of Pharmaceutical Sciences, Naresuan University, Thailand.