CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

All chemicals used in this study were analytical grade and listed as following :

Chemical Reagents	Sources
1. Absolute ethanol (C_2H_5OH)	E. Merck, Germany
2. Acetic acid gracial (CH_3COOH)	E. Merck, Germany
3. Alumina (Al_2O_3)	Sigma chemical Co., USA
4. Acetylacetone ($CH_3COCH_2COCH_3$)	Sigma chemical Co., USA
5. Ammonium acetate (CH_3COONH_4)	E. Merck, Germany
6. Bovine serum albumin (BSA)	Sigma chemical Co., USA
7. Calcium chloride dihydrate ($CaCl_2.2H_2O$)	E. Merck, Germany
8. Ca^{2+} ionophore (A23187)	Sigma chemical Co., USA
9. Diethyl ether [$(C_2H_5)_2O$]	Lab scan Asia Ltd., Thailand
10. Disodium hydrogen phosphate (Na_2HPO_4)	E. Merck, Germany
11. Glucose anhydrous	Sigma chemical Co., USA
12. Glycine ($C_2H_5NO_2$)	Sigma chemical Co., USA
13. Hemoglobin Standard	Sigma chemical Co., USA
14. Heparin (sodium salt)	Sigma chemical Co., USA
15. Hydrazine hydrate ($N_2H_4.H_2O$)	Sigma chemical Co., USA
16. Isopropanol [$(CH_3)_2CHOH$]	E. Merck, Germany
17. L (+)Lactic acid ($CH_3CH(OH)CO_2H$)	E. Merck, Germany
18. Lactate dehydrogenase (Type III, from beef heart)	Sigma chemical Co., USA
19. Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	APS Finechem, Australia

Chemical Reagents	Sources
20. Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	E. Merck, Germany
21. Metaphosphoric acid [$(HPO_3)n$]	Riedel-de Haen AG, Germany
22. Nicotinamide adenine dinucleotide (NAD^+)	Sigma chemical Co., USA
23. Ortho-toluidine (*o*-toluidine)	E. Merck, Germany
24. Potassium ferricyanide [$K_3Fe(CN)_6$]	E. Merck, Germany
25. Potassium Cyanide (KCN)	E. Merck, Germany
26. Potassium oxalate monohydrate ($C_2O_4K_2.H_2O$)	Sigma chemical Co., USA
27. Potassium hydroxide (KOH)	E. Merck, Germany
28. Potassium chloride (KCl)	E. Merck, Germany
29. Potassium dihydrogen phosphate (KH_2PO_4)	E. Merck, Germany
30. Sigma kit 35-UV (for 2,3-DPG determination)	Sigma chemical Co., USA
30.1 Triethanolamine buffer solution	
30.2 Nicotinamide adenine dinucleotide, reduced form (NADH)	
30.3 Adenine 5'-Triphosphate (ATP)	
30.4 Phosphoglycerate mutase (PGM)	
30.5 GAPD/PGK enzyme mixture	
30.6 Phosphoglycolic acid	
30.7 Trichloroacetic acid solution	
31. Sodium chloride (NaCl)	E. Merck, Germany
32. Sodium fluoride (NaF)	E. Merck, Germany
33. Sodium hydroxide (NaOH)	E. Merck, Germany
34. Sodium hydrogen carbonate ($NaHCO_3$)	E. Merck, Germany

Chemical Reagents	Sources
35. Sodium periodate ($NaIO_4$)	BDH Chemicals Ltd., England
36. Sodium sulphate (Na_2SO_4)	E. Merck, Germany
37. Thiourea (H_2NCSNH_2)	Riedel-de Haen AG, Germany
38. Triolein ($C_{57}H_{104}O_6$)	Fluka Chemika, Switzerland

2.2 Instruments

Instrument	Model	Sources
1. Centrifuge	H-103 N	Kokusan, Tokyo Japan
2. High speed microcapillary Hematocrit centrifuge	D-7200	Hettich, Germany
3. pH meter	pH 538	TUV, Germany
4. Spectrophometer	UV-2410 PC	Shimadzu, Japan
5. Hemocytometer		
6. Microscope	CH3 ORF200	Olympus, Japan
7. Vortex mixture		
8. Temperature controlled water bath		
9. Hot plate stirrer	HPMS	Whatman, England
10. Refrigerator (-80^{O}C)		
11. Motorized rodent treadmill		Columbus, USA
12. Blood gas analyzer	ABL 555	Japan

2.3 Animal care

Male Wistar rats, approximately 200-220 g body weight, were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The rats were allocated in nine groups of 6 animals, and were individually housed in stainless steel suspended cages, provided with food and water *ad libitum*. Rats were acclimatized to temperature (26 ± 1°C), humidity (50 ± 10%) and light (12 hr dark/light cycle: light off at 7 : 00 AM) for a period of 1 week after their arrival, and then were introduced to treadmill running. This initial treadmill exposure amounted to running 15 minutes daily at 20 m/min for 5 days. Rats readily adjusted to running procedure (approximately 90%) were assigned to one of the running groups or kept sedentary (Dunley et al., 1982).

2.4 Training regimens

Rats were randomly allocated into nine groups of 6 animals. Groups 1-3 were limited to cage activity or kept sedentary for 2,5 and 8 weeks, respectively. Group 4 was subjected to running by endurance training program (Table 2-1) for 5 days per week. The training was at a speed of 20 m/min and a slope of 0° for 15 min/day in the thoroughness of the first week. After, a speed was increased to 28 m/min and a slope of 5° for 60 min/day during the second week. In the third week, a speed at 28 m/min and a slope of 10 for 60 min/day were maintained until the training was terminated. Group 5 and 6 also ran by endurance training program for 5 and 8 weeks, respectively. Group7 was subjected to running by exhaustion training program for 2 weeks. All animals had daily running 5 days per week and the training program was initiated after a 5 minutes warm up at 10.7 m/min on a 15° incline, the treadmill speed was increased by 5.4 m/min every 2 min until the rats were exhausted. Group 8 and 9 were ran by exhaustion training program (Table 2-2) for 5 and 8 weeks, respectively.

Table 2-1: Summary of endurance training program (Helge *et al.*, 1998).

Week	Speed (m/min)	Slope of treadmill track (degree)	Running time (min/day)
1	20	0	15
2	28	5	60
3-8	28	10	60

Table 2-2: Summary of exhaustion training program (Pels *et al.*, 1995).

Time (min)	Speed* (m/min)	Slope of treadmill track (degree)
1-5 (warm up)	10.7	15
7	16.1	15
9	21.5	15
11	26.9	15

* Speed was increased by 5.4 m/min every 2 minutes until exhaustion.

2.5 Blood collection

Immediately after running or exhaustion, exercised rats and matched sedentary control were subsequently anesthetized using diethylether and blood samples were collected by cardiac puncture. Potassium oxalate was used as an anticoagulant in this experiment. Whole blood was mixed by inverting the tube four or five times and kept in an ice bath for subsequent assay of 2,3-BPG, lactate, glucose, triglyceride and hematological parameters (Figure 2-1).

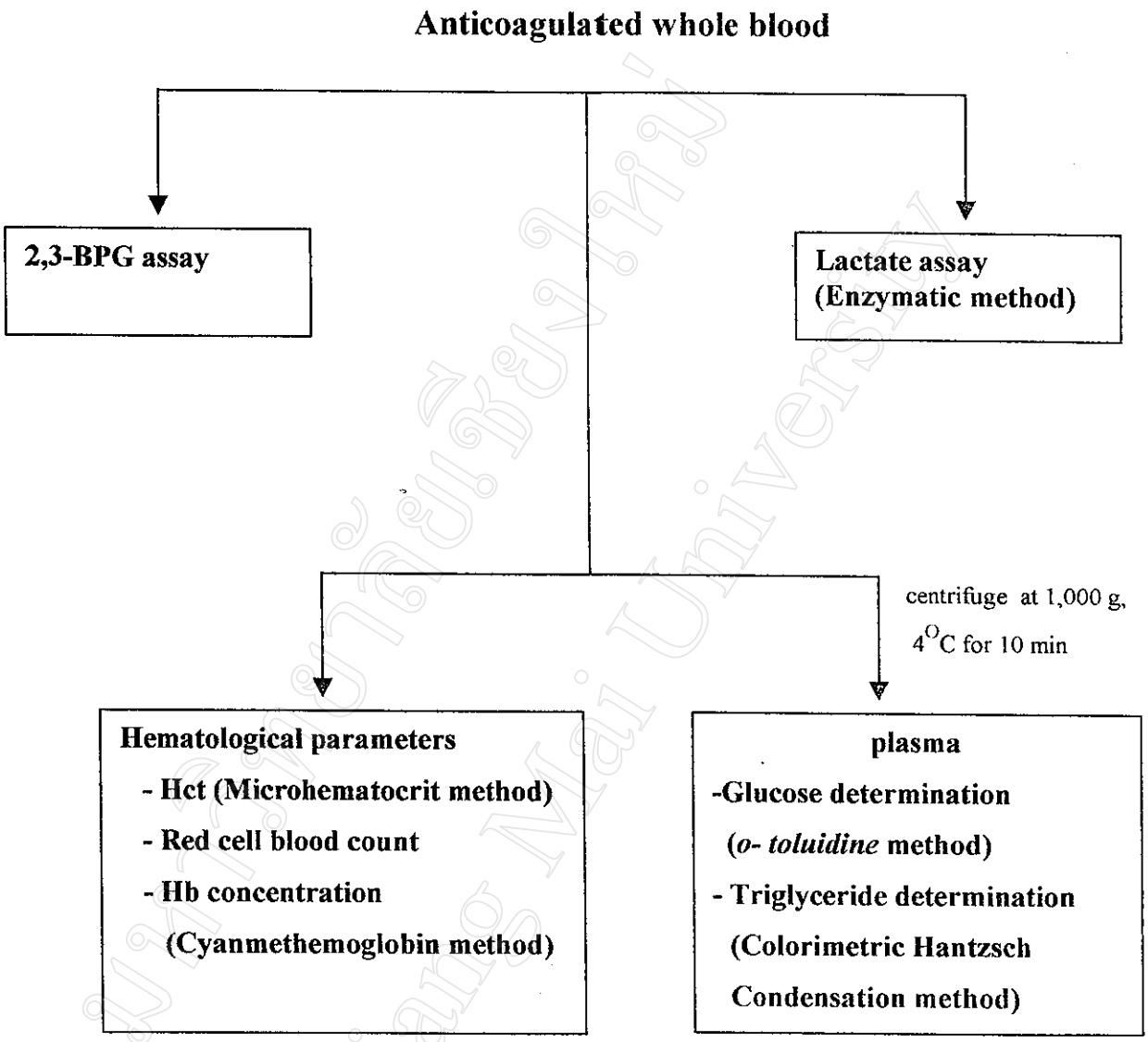

Fig 2-1: Schematic representation of the procedure in this experiment.

2.6 2,3-Bisphosphoglycerate assay

The assay was performed according to the method shown in Sigma Kit 35-A from Sigma Chemical Co., St. Louis and Sigma procedure No.665

Principle

The three enzymatic reactions involving in the described procedure are as follows :

1. 2,3-BPG is hydrolyzed to 3-PG and inorganic phosphate by 2,3-BPG phosphatase. 2-Phosphoglycolate is needed as a stimulator for this reaction.

$$\text{2,3-BPG} \xrightarrow[\text{2-Phosphoglycolate}]{\text{BPGP}} \text{3-PG + Pi}$$

2. 3-PG reacts with ATP in the presence of PGK to form 1,3-BPG and ADP.

$$\text{3-PG + ATP} \xrightarrow{\text{PGK}} \text{1,3-BPG + ADP}$$

3. 1,3-BPG is oxidized by GA-3-PD in the presence of NADH to produce to GA-3P and NAD^+.

$$\text{1,3-BPG + NADH} \xrightarrow{\text{GA-3-PD}} \text{GA-3P + } NAD^+$$

Measurement of the decrease in the absorbance of NADH at 340 nm reflects the amount of 2,3-BPG originally present.

Reagents

1. Triethanolamine buffer
2. NADH
3. ATP
4. PGM
5. GA-3PD/PGK mixture
6. 2-phosphoglycolate
7. TCA 8% (w/v)

Procedure

1. Pipet 1.0 ml of fresh blood into 3.0 ml of 8% TCA (w/v). Shake vigorously for several seconds. Keep the mixture on ice-bath for an additional 5 minutes to assure complete protein precipitation.
2. Centrifuge 10 minutes at 1,000 g to obtain clear supernatant.
3. Into a 1-mg NADH vial, pipette 8.0 ml Triethanolamine buffer solution. Cap and invert several times to dissolve the NADH. This solution is sufficient for 3 tests and stable up to one week when stored in refrigerator (2-8 ^{O}C).
4. Into a cuvet, pipet 2.5 ml solution from step 1, 0.1 ml ATP solution and 0.25 ml protein-free supernatant were added and mixed by inversion.
5. Then, add 0.02 ml GA-3PD/PGK mixture and 0.02 ml PGM. Mix by inversion. Wait 5 minutes. (Any delay beyond 5 minutes will cause an underestimation of 2,3-BPG content.)
6. Read and record absorbance at 340 nm vs water as reference. This is INITIAL A.
7. Add 0.1 ml 2-phosphoglycolate and mix by inversion.
8. Let stand for 30 minutes at 25^{O}C or 15 minutes at 37^{O}C to allow reaction to go to completion
9. Read and record absorbance at 340 nm. vs water as reference. This is FINAL A.

NOTE: The FINAL A is constant for at least 15 minutes.

The whole procedure is shown by a flow chart in Figure 2.2

Calculation:

ΔA = INITIAL A - FINAL A

Corrected ΔA = ΔA - 0.03

Blood 2,3-BPG (μmol/ml) = Corrected ΔA x 7.7

NOTES: 1. When corrected ΔA exceeds 0.550 using a 1-cm cuvet (2,3-BPG level greater than 4.2 μmol/ml), repeat assay using 0.1 ml supernatant and 0.15 ml water. Multiply the result by 2.5.

2. The value 0.030 is subtracted from the ΔA to correct for the change in volume due to addition of 2-phosphoglycolate and has been determined empirically.

3. The factor is derived as follows: 2.99 / (6.22 x 0.0625) = 7.7

Where: 2.99 = volume(ml) of reaction mixture in cuvet

6.22 = absorbance at 340 nm of a solution containing 1 μmol NADH/ml.

0.0625 = volume (ml) of original sample in reaction mixture

Expression of the results in terms of packed cells (μmol/ml):

$$\text{Packed cell 2,3-BPG (μmol/ml)} = \frac{\text{Blood 2,3-BPG(μmol/ml) x 100}}{\text{Hematocrit (\%)}}$$

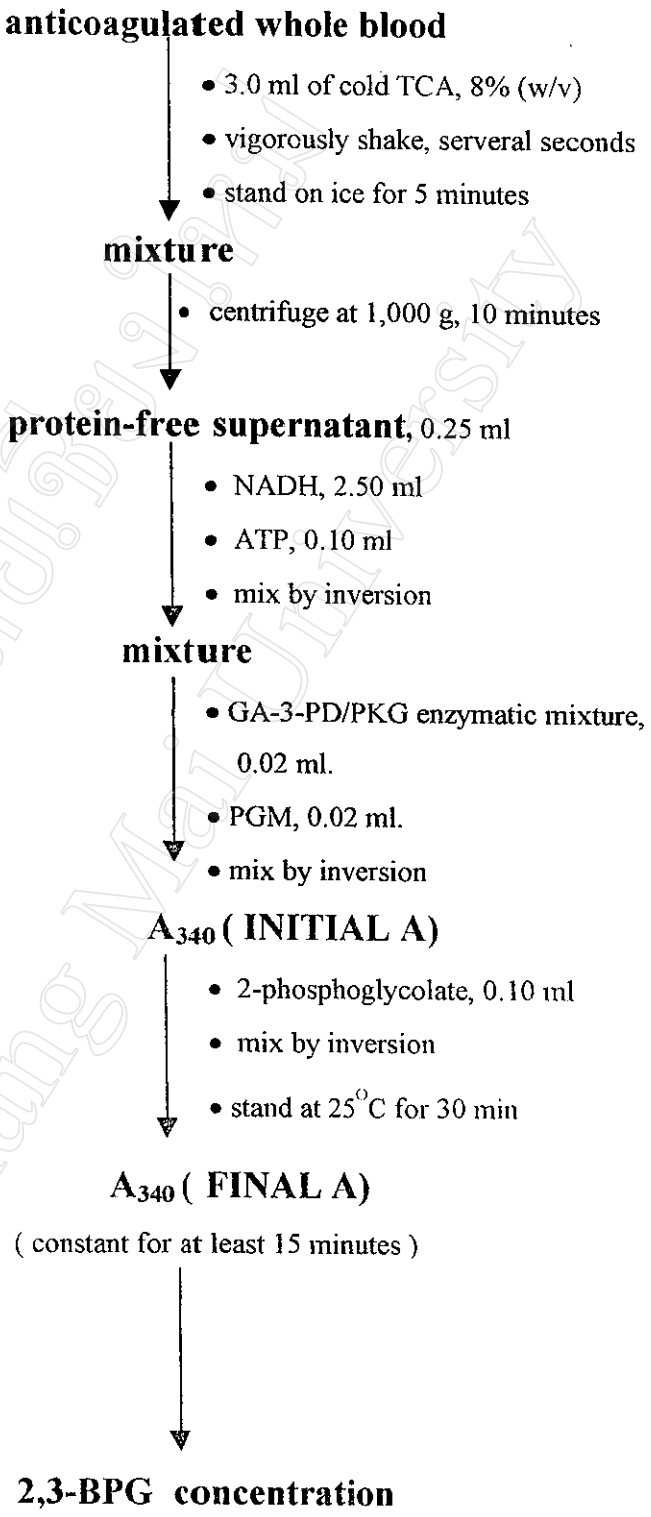

Figure 2-2: The whole procedure of 2,3-BPG assay.

2.7 Determination of hematological parameters

2.7.1 Determination of hematocrit by microhematocrit method

Principle

Whole blood is centrifuged for maximal red blood cell packing. The space occupied by the red blood cells is measured and expressed as a percentage of the whole blood volume.

Procedure (Dacie and Lewis, 1968)

1. Allow well mixed anticoagulated whole blood to enter two microhematocrit tubes 7 cm in length and 1 mm core until approximately two-third is filled with blood without air bubbles.
2. Seal one end of the microhematocrit tube with clay material by placing the dry end of the tube into clay in a vertical position. The plug should be 4 to 6 mm long. Notice that certain blood is not forced out the top of the hematocrit tube during this process.
3. Place the two microhematocrit tubes in the radial grooves of the centrifuge head exactly opposite each other, with the sealed end away from the center of the centrifure.
4. Centrifuge 11,500-15,000 rpm for 5 minutes.
5. Remove the hematocrit tubes as soon as the machine has stopped spinning. Obtain the results from both microhematocrit tube. Results should agree within $\pm$ 2% of the hematocrit result. If they do not, repeat the preceding procedure.
6. The percent hematocrit is calculated by the follow equation.

$$\text{\% Hematocrit} = \frac{\text{packed cell volume in the length} \times 100}{\text{total volume in the length}}$$

2.7.2 Determination of hemoglobin by cyanmethemoglobin method

Principle

Whole blood is added to cyanmethemoglobin reagent (Drabkin's solution). The ferricyanide converts the hemoglobin iron from the ferrous state (Fe^{2+}) to the ferric state (Fe^{3+}) to form methemoglobin (metHb) which then combines with potassium cyanide to form stable pigment, cyanmethemoglobin (HbCN; hemoglobin cyanide). The color intensity of this mixture is measured in a spectrophotometer at a wavelength of 540 nm. The optical density of the solution is proportional to the concentration of hemoglobin.

Reagents

Drabkin's solution

$K_3Fe(CN)_6$	0.1	g
KCN	0.025	g
$NaHCO_3$	0.5	g
distilled water up to	500	ml

Mix thoroughly and store in a brown bottle at room temperature. The solution is stable for one month.

Procedure (Brown, 1988)

1. For each sample to be tested, pipette exactly 5.0 ml of Drabkin's solution into an appropriately labeled test tube. Place 5.0 ml of the reagent into a test tube to be used as the blank.
2. Add 0.02 ml. of well mixed whole blood to the appropriately labeled tube. Rinse the pipet 3 to 5 times with the Drabkin's solution until all blood is removed from the pipet.
2. Mix the preceding solutions well and allow to stand at room temperature for at least 10 minutes in order to allow adequate time for the formation of HbCN.

4. Transfer the mixture to the cuvet and read the optical density in a spectrophomometer at a wavelength of 540 nm. Using the Drabkin's solution in the blank tube to set zero optical density (A_{540}). Record the readings for the samples from the absorbance scale and refer to the standard curve for actual value of the hemoglobin in g/dl.

Preparation of standard curve for hemoglobin

Using the stock solution of HbCN standard (equivalent to18 g hemoglobin per 100 ml blood), set up the dilutions to 3.6, 7.2, 10.8, 14.4 and 18 g (Hb)/dl. Plot hemoglobin in g/dl on the horizontal axis against A_{540} on the vertical axis, this is a straight-line curve. A chart should then be made to facilitate reading the test results.

Table 2-3: Dilutions and A_{540} for hemoglobin standard curve

Tube No.	Standard Hb (ml)	Drabkin's solution (ml)	Hb (g/dl)	A_{540}
1.	5	0	18.0	0.47
2.	4	1	14.4	0.37
3.	3	2	10.8	0.28
4.	2	3	7.2	0.19
5.	1	4	3.6	0.09
6.	0	5	0.0	0.0

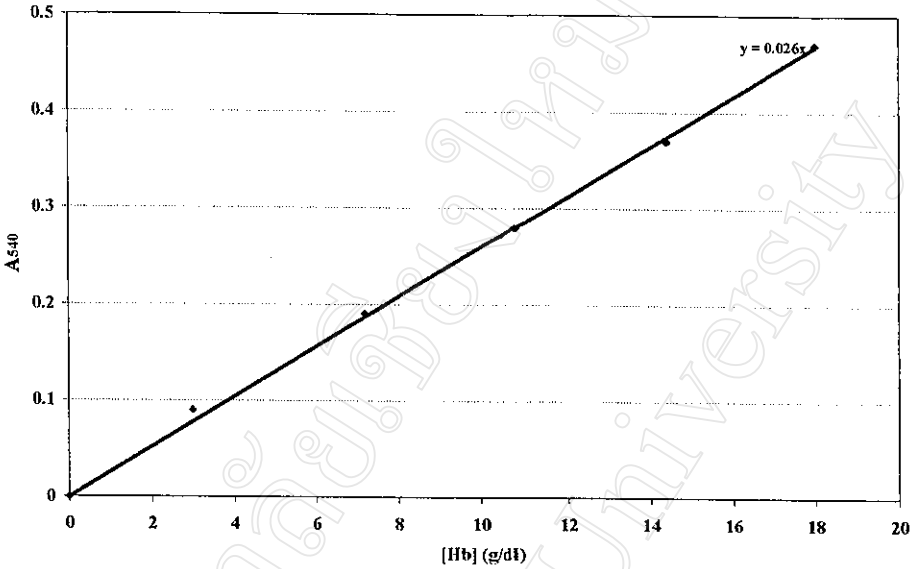

Figure 2-3: Hemoglobin standard curve

2.7.3 Red blood cell count

Principle

The red blood cell count (RBC) is the number of red blood cells in 1 cu. mm of whole blood. Blood is diluted with an isotonic fluid. The diluted specimen is introduced into the hemocytometer chamber, and the erythrocytes are counted. Diluting fluids used for erythrocyte counts do not destroy the leukocytes. There are normally so few that they do not interfere with the enumeration of the erythrocytes. When there are many, they are easily identified and are not counted.

Reagents

Grower's solution

Na_2SO_4	12.5 g
glacial acetic acid	33.3 ml
distilled water up to	200 ml

Mix and store at room temperature.

Procedure (Brown, 1988)

1. Dilute 2 µl blood with 400 µl of diluting fluid (Grower's solution).
2. Fill both sides of the clean hemocytometer chamber.
3. Count the red blood cells as described in the following step
   a. Carefully place the filled counting chamber on the microscope stage.
   b. Using low power (10x objective), place the large center square in the middle of the field of vision. Carefully examine the entire large square for even distribution of red blood cells.
   c. Carefully change to the high dry 40 x objective.

d. Move the counting chamber so that the small upper left corner square is completely in the field of vision. This square is further subdivided into sixteen even smaller squares. This facilitates cell counting.

e. Count the red cell in the small square. Remember to count the cells on two of the outer margins but exclude those lying on the other two outside edges.

f. Some of the red blood cells may be lying on their side and, there for, do not appear as round as the majority of cells in the area. These cells are to be included in the count.

g. White blood cells, if presents in the counting area are not included in the count.

h. Count the red blood cells on the opposite site of the counting chamber in the corresponding center square.

4. Calculate the red blood cell count for each of the red cell counts performed and average the two results for the final report. The equation for calculation of the red blood cell count is shown as following.

RBC/cu. mm	=	**RBC in five squares**	x	**correction for volume**	x	**correction for dilution**

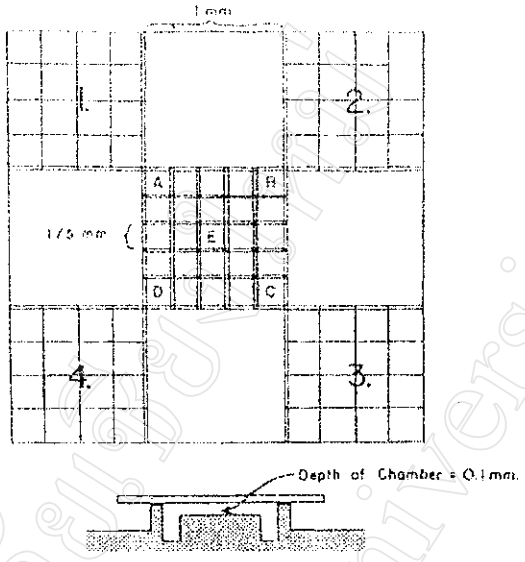

Figure 2-4: Hemocytometer squares 1,2,3 and 4 are used for counting leukocytes; squares A, B, C, D and E are used for counting erythrocytes (Brown, 1988).

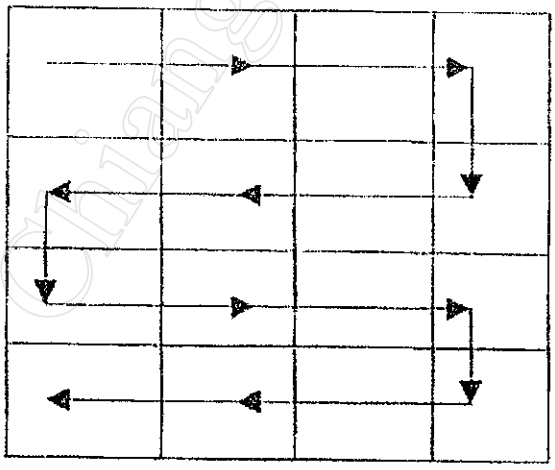

Figure 2-5: Manner of counting erythrocytes in one of small squares (Brown, 1988).

2.8 The effects of anticoagulants on erythrocyte 2,3-BPG preservation

2.8.1 The effect of anticoagulants on erythrocyte 2,3-BPG levels in blood.

To study the effectiveness of anticoagulants as a preservative for erythrocyte 2,3-BPG levels, heparin, potassium oxalate and sodium fluoride were used as anticoagulants.

A known amount of potassium oxalate or NaF were dissolved in distilled water to get 1g% and 3g%, respectively. Then, these solutions were added to the test tubes and dried in an hot air oven at 70°C.

Blood samples were obtained from 5 normal male Wistar rats (325 ± 25 g) and aliquoted each sample into three sets of tubes containing heparin (50 unit/ml blood) (Sigma Technical Service); potassium oxalate (2 mg/ml blood) (Oser, 1965) or NaF (6mg/ml blood) (Chan *et al.*, 1992). The contents of each tube were gently mixed. One set was precipitated with cold TCA (8% w/v) immediately and the protein-free supernatant was assayed for 2,3-BPG. The remaining sets were stored at 4°C for 5 days and the protein-free supernatant was prepared for erythrocyte 2,3-BPG assay. The hematocrit was analyzed in each sample.

2.8.2 The effects of storage on erythrocyte 2,3-BPG levels when potassium oxalate was used as an anticoagulant.

To study the effects of potassium oxalate on erythrocyte 2,3-BPG levels of blood stored at 4°C for 5, 10, 15, 20, 25 and 30 days. Blood samples were obtained from 5 normal male Wistar rats (325 ± 25g) and aliquoted into seven sets of tubes containing potassium oxalate. The contents of each tube were gently mixed. The erythrocyte 2,3-BPG concentrations were immediately assayed in one set. The remaining sets were stored at 4°C and precipitated with cold TCA (8% w/v) to provide protein-free supernatants for 2,3-BPG assay. The hematocrit was analyzed in each sample.

2.9 Determination of L-lactate by enzymatic method

Principle

L-lactate is converted to pyruvate by lactate dehydrogenase using NAD^+ coenzyme in the reaction

$$\text{Lactate} + NAD^+ \xrightleftharpoons{\text{LDH}} \text{pyruvate} + NADH$$

The quantity of measured lactate is the appearance of the reduced coenzyme NADH as determined by an increase in absorbance at 340 nm.

Reagents

1. 0.5 M Glycine- 0.4 M hydrazine buffer

Glycine	37.5 g
Hydrazine hydrate	20 ml
distilled water approximately	300 ml

The pH was adjusted to 9.0 at room temperature with NaOH. The solution was diluted to 1,000 ml with distilled water. Mix thoroughly and store in a brown bottle at room temperature.

3. LDH solution
4. 0.027 M NAD^+ solution
5. 5% (w/v) meta-phosphoric acid
6. 0.1 M L-lactate standard solution

Procedure (Marbach and Weil, 1967)

1. Exactly 0.5 ml of freshly whole blood was delivered to a test tube containing 1.5 ml of cold 5% meta-phosphoric acid. The test tube was quickly stoppered and the contents were mixed thoroughly by inversion and allowed to stand for 5 minutes.
2. Centrifuge 10 minutes at approximately 1,000 g to obtain clear supernatant.
3. To a cuvet containing 2 ml glycine-hydrazine buffer, 0.1 ml of supernatant was added. The reagent was mixed by a plastic stick and 0.030 ml of LDH solution and 0.20 ml NAD^+ were then added.
4. After remixing, the contents were left to stand at ambient temperature. After 14 min, the absorbance was read at 340 nm against water blank. In the blank, 0.1 ml of 3% meta-phosphoric acid was substituted for the supernatant.
5. The absorbance due to lactate was corrected by substraction of the absorbance of the blank from the standard or sample test.
6. The results were calculated by the follow equation:

Calculation:

$$\text{Lactate (mM)} = \frac{(A_{sample} - A_{blank})}{(A_{standard} - A_{blank})} \times \text{Standard conc.} \times D$$

Where A is the absorbance, ΔA is the change in absorbance, and D is the dilution of blood sample (in protein-free supernatant).

When exactly 0.5 ml of blood was added to 1.5 ml of 5% meta-phosphoric acid, D = 4. When approximately 1 ml of blood is added to tared test tube containing exactly 3 ml of 5% meta-phosphoric acid:

$$D = \frac{(\text{ml of blood added} + 3)}{\text{ml of blood added}} \times 1.06$$

2.10 Glucose determination by *O*-toluidine method

Principle

When heated with glucose, a solution of the aromatic amine *o*-toluidine in strong acidic solution produces a color product with an absorption maximum at about 630 nm. When condensing to the reagent, the aldehyde group of glucose first forms a glycosyl amine intermediate, then rearranges to from a Schiff base color product (Figure 2-6)

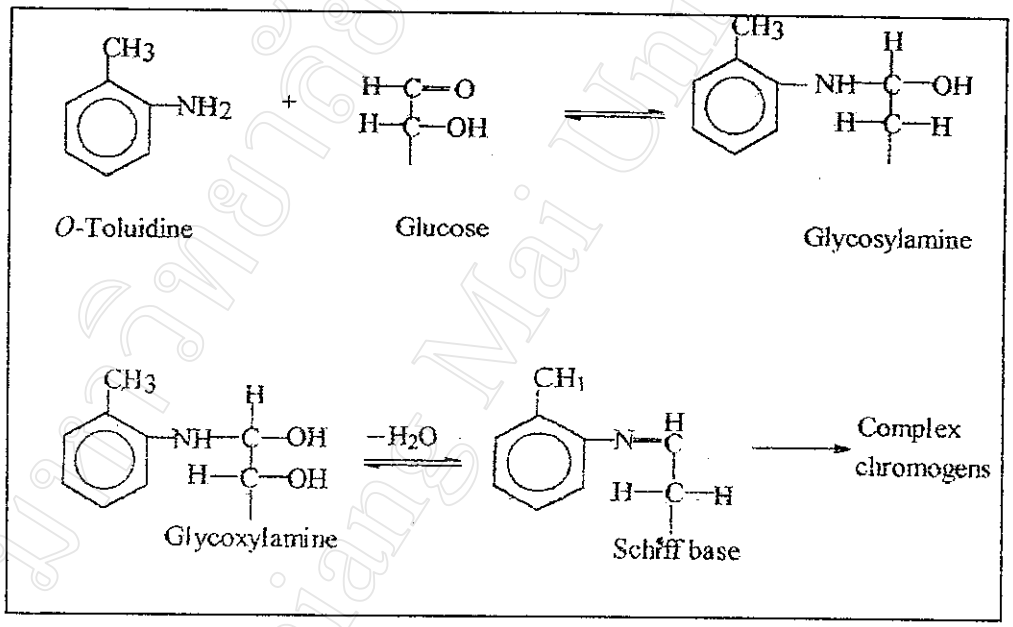

Figure2-6: Schiff base reaction between *o*-toluidine and aldehyde group of glucose.

Reagents

o-toluidine reagent

Thiourea	1.5 g
glacial acetic acid	940 ml
o-toluidine	60 ml

Mix and store at room temperature.

Procedure (Bauer, 1982)

1. Pipetted 3.0 ml of *o*-toluidine reagent in a series of glass tube, preferably, one with Teflon-lined screw caps.
2. With accurate micropipet added separately to the tubes 50 µl aliquots of the standard and unknown sample, also reserved one tube of *o*-toluidine reagent alone as a blank.
3. Mixed the contents of each tube, capped, and heated tubes in a boiling water bath for 12 min.
4. The tubes were removed from heat, cool in ice water, then brough to room temperature, and read optical density of standards and samples against blank at 630 nm.
5. Calculated the results by the following equation:

Calculation:

$$\text{Conc. of sample} = \frac{A_{sample}}{A_{standard}} \times \text{Conc. of standard}$$

Thus :

$$\text{Glucose (mg)/ sample (dl)} = \frac{A_{sample}}{A_{standard}} \times 100$$

2.11 Determination of triglyceride by colorimetric Hantzsch condensation method

Principle

This measurement is based on the original method of Fetcher., et al(1968). The modified method for determining triglycerides in serum, in which alumina is used to adsorb interfering substances (such as phospholipids, glycerol and glucose from the propanol extract), and the glycerol content of triglycerides is determined colorimetrically. Serum triglycerides are commonly measured by saponifying triglycerides to glycerol, which is oxidized by sodium metaperiodate to formaldehyde. Formaldehyde is condensed with acetylacetone and ammonia to form 3,5-diacetyl-1,4-dihydrolutidine (Hantzsch condensation), the sequential reaction is shown in figure 2-7. It is the final chromogen with an absorption maximum at 405 nm that is proportional to the concentration of serum triglyceride.

Reagents

1. Saponification reagent

KOH	50 g
distilled water	600 ml
Isopropanol up to	1,000 ml

Mix and store at room temperature.

2 Acetylacetone reagent

Acetylacetone	7.5 ml
Isopropanol	200 ml
distilled water up to	1,000 ml

Mix and store at room temperature

3. Sodium metaperiodate

Anhydrous ammonium acetate	77	g
Sodium metaperiodate	0.650	g
distilled water	700	ml
glacial acetic acid	60	ml

The solution is diluted to 1L with distilled water. Then, mix and store at room temperature.

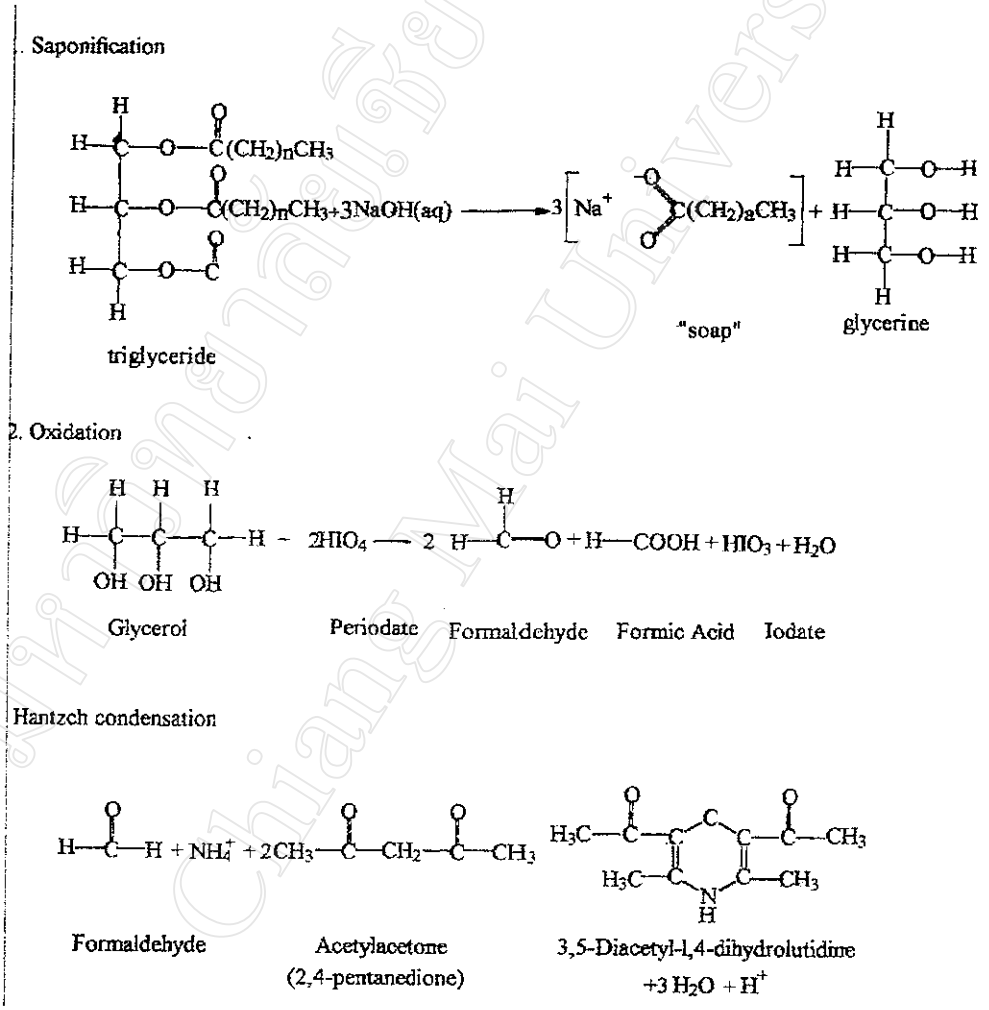

Figure 2-7: The sequential reaction in colorimetric Hantzsch condensation.

Procedure (Foster and Dunn, 1973)

1. To appropriate screw-capped glass tubes, added 0.1 ml of water, unknown serum or standard.
2. Added 4.0 ml of isopropanol and mixed well.
3. Added 0.4 g of washed alumina (a "calibrated scoop" may be used) to all tubes and placed them on a mechanical rotator for 15 min.
4. Centrifuged at 1,000g for 10 min to separate the alumina from the solutions and pipetted 2.0 ml of the supernatant to appropriately labeled test tubes or cuvets.
5. Added 0.6 ml of saponification reagent (alcolic KOH) to all tubes, mixed on a vortex-type mixer. Tightly covered each tube with "parafilm" and placed all tubes in a 65 $\pm$ 2°C water bath for at least 15 min.
6. Removed all tubes from the water bath and allowed to cool to room temperature. Added 0.5 ml of sodium metaperiodate reagent, mixed on a Vortex-type mixer and allowed to stand at room temperature for at least 15 min.
7. Then, added 0.5 ml of acetylacetone raegent and mixed on a Vortex-type mixer. After that, the pH value was measured by lismus paper. The pH of reaction mixture was adjusted with 0.1 M HCl when it was not in the normal range 6.05 $\pm$ 0.1. Tightly covered tube with "parafilm" and placed all tubes in 65 $\pm$ 2°C water bath at least 15 minutes .
8. Removed all tubes from the water bath and allowed them to cool to room temperature. Measured the absorbance of standard and unknown against the blank at 405 nm.
9. The results were calculated by the following equation:

Calculation

$$\text{Triglyceride (mg\%)} = \frac{A_{sample}}{A_{standard}} \times \text{Standard conc. (mg\%)}$$

2.12 Factors influencing erythrocyte 2,3-BPG levels.

2.12.1 Animals and preparation of red blood cells.

Male Wistar rats, weighing approximately 350 ± 20 g, were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. Rats were not subjected to exercise. Blood sample was drawn by cardiac puncture with potassium oxalate anticoagulant. Anticoagulated whole blood was centrifuged at 1,000 g, 4°C for 10 min. The plasma and buffy coat were removed by aspiration and the packed red cells were washed three times with normal saline and then resuspended with Hank Balance Salt solution (HBSS) at approximately 30% hematocrit (see Figure 2-8).

Reagents

1. 0.9% NaCl
2. Ca^{2+} ionophore (A23187)
3. HBSS

Glucose anhydrous	1 g	
NaCl	8.0004 g	136.9 mM
KCl	0.4026 g	5.4 mM
Na_2HPO_4	0.0468 g	0.3 mM
KH_2PO_4	0.0408 g	0.3 mM
$MgSO_4.7H_2O$	0.0986 g	0.4 mM
$CaCl_2$	0.1911 g	1.3 mM
$MgCl_2$	0.1017 g	0.5 mM
$NaHCO_3$	0.3528 g	4.2 mM
distilled water up to pH 7.4	1000 ml	

2.12.2 The effect of pH on erythrocyte 2,3-BPG levels

The procedure was as follow:

1. The packed red cells were resuspended with HBSS pH 6.4, 6.6, 6.8, 7.0, 7.2 at approximately 30% hematocrit. Each suspension was matched with the packed red cells suspension HBSS pH 7.4.
2. Pipetted 0.5 ml of the red cell suspension into a microcentrifuge tube and incubated all tubes in 37°C water bath for 15 minutes.
3. Removed all tubes from the water bath and immediately, pipetted 0.2 ml of incubated red cell suspension into 0.6 ml of 8% TCA (w/v). The protein-free supernatant, obtained by speed centrifugation at 1,000 g 4°C for 10 min, was collected for 2,3-BPG assay.
4. 2,3-BPG was assayed using Sigma kit 35-UV for 2,3-BPG determination.
5. The diagram below shows the whole procedure.

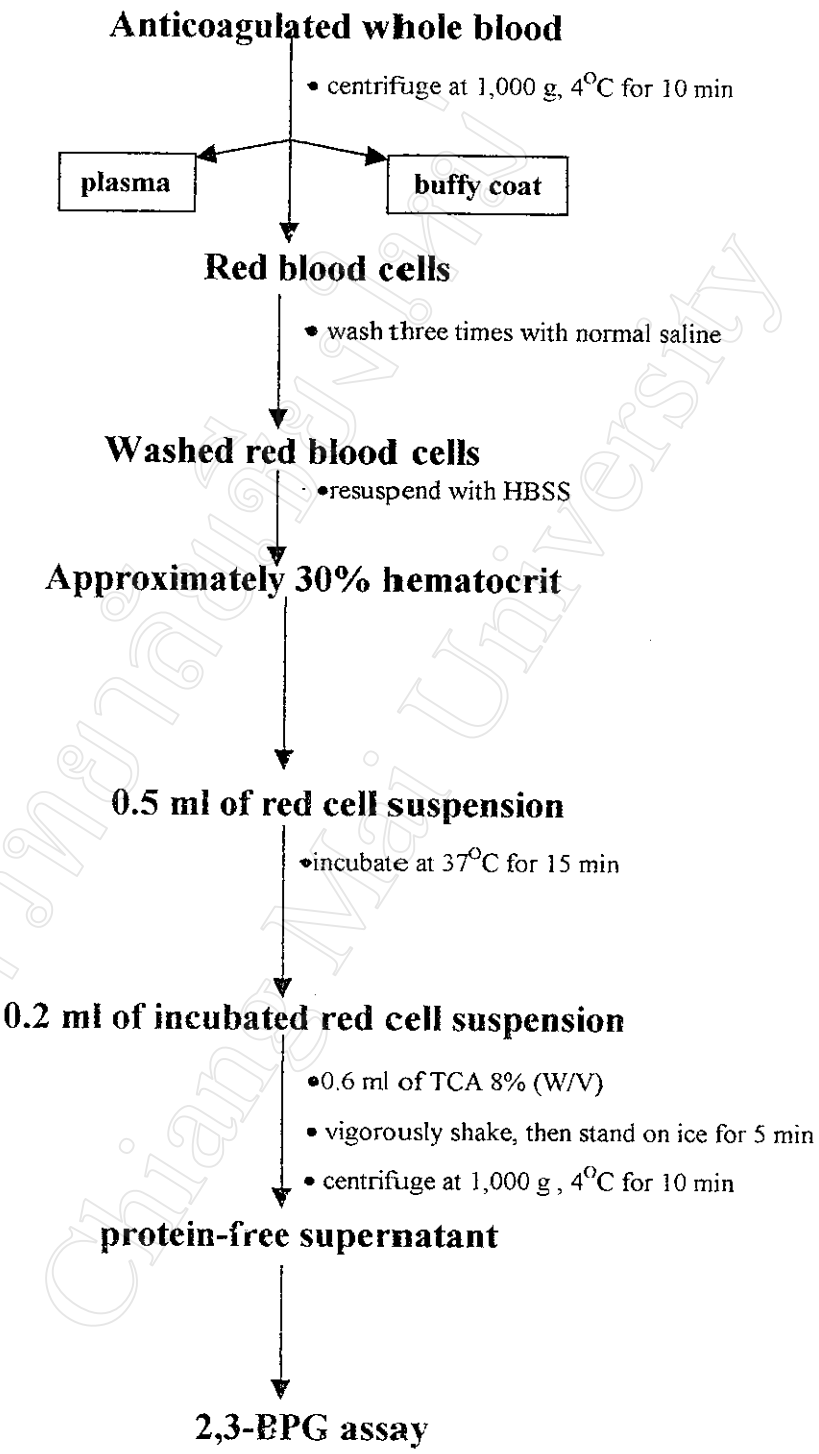

Figure 2-8: The common procedure in the experiments studying the factors influencing erythrocyte 2,3-BPG levels.

2.12.3 The effect of temperature on erythrocyte 2,3-BPG levels.

The procedure was as follow:

1. The packed red cells were resuspended with HBSS pH 7.4 at approximately 30% hematocrit.
2. Pipetted 0.5 ml of the red cell suspension into a microcentrifuge tube and placed all tubes at 35°C, 39°C, 41°C, 43°C and 45°C in water bath for 30 min, each tube was matched with physiological temperature (37°C).
3. Remove all tubes from the water bath and immediately, pipetted 0.2 ml of incubated red cell suspension into 0.6 ml of 8% TCA (w/v). The protein-free supernatant, obtained by speed at 1,000 g, 4°C for 10 min, was collected for 2,3-BPG assay.

2.12.4 The effect of calcium ionophore (A23187) on erythrocyte 2,3-BPG levels.

A23187 was used as a tool to study the effect of Ca^{2+} on erythrocyte 2,3-BPG levels. A23187 is a carboxylic acid antibiotic that is crystallized from broths of *Streptomyces chartreusensis*. It has a molecular weight of 523, an elemental analysis is $C_{29}H_{37}N_3O_6$ (Reed and Lardy, 1972). A23187 is a lipid soluble molecule that complexes alkali metal cations and transport them across a variety of membranes or into a bulk organic phase, and has a high selectivity for divalent over monovalent ions. This can be attributed to the probable inclusion of two nitrogen atom ligands in the complexation sphere (Pressman, 1976) (Figure 2-8). This structure is based on X-ray crystallography of uncomplexed A23187 and so it is not possible to be certain which oxygens and nitrogens ligand in complexs. Thus it is capable of stimulating various Ca^{2+}-dependent biological reactions without disturbing preexisting balance of Na^+ and K^+. It also transports Mg^{2+}, but gradients of this ion across biological membrane seldom participate in biological control. Thus, A23187 is Ca^{2+} ionophore (Pressman, 1976).

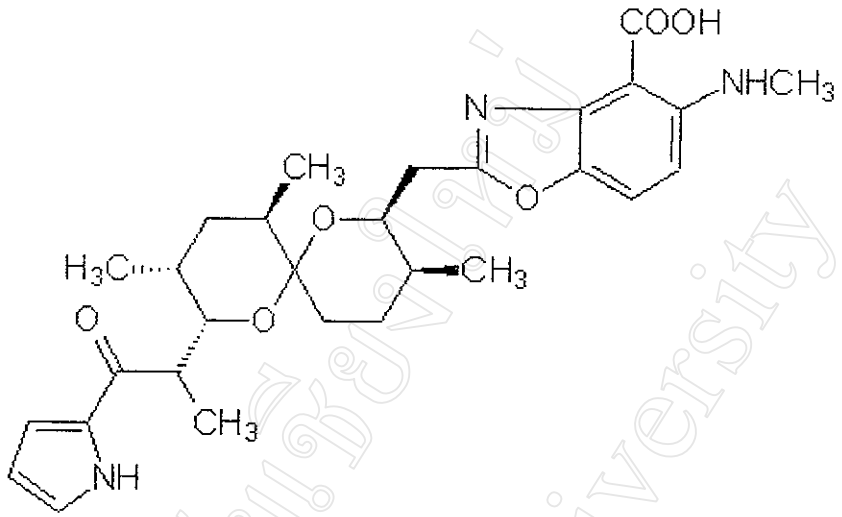

Figure 2-9: Structure of calcium ionophore A23187 (Pressman, 1976).

Procedure was as follow:

1. The packed red cells were resuspended with HBSS pH 7.4 at approximately 30% hematocrit.
2. Pipetted 0.5 ml of red cell suspension into a microcentrifuge tube that containing 1.25 µl, 2.5 µl, 3.75 µl, 5 µl or 7.5 µl of 1.00 mM A23187 and not containing A23187 to served as control. All tubes were gently mixed and incubated in a 37°C water bath for 15 minutes.
3. Removed all tubes from the water bath and immediately pipetted 0.2 ml of incubated red cell suspension into 0.6 ml of 8% TCA (w/v). The protein-free supernatant, obtained by speed centrifugation at 1,000 g, 4°C for 10 min, was collected for 2,3-BPG assay.

2.12.5 The effect of pO_2 on erythrocyte 2,3-BPG levels.

The procedure was as follow:

1. The packed red cells were resuspended with HBSS pH 7.4
2. To appropriately capped glass tubes, aliquots of 0.5 ml of the red cell suspension were added. Each tube was blown with nitrogen gas for 5-15 minutes and then sealed with a cap. All tubes were placed in 37°C water bath for 15 minutes.
3. Removed all tubes from the water bath and immediately pipetted 0.2 ml of incubated red cell suspension into 0.6 ml of 8% TCA (w/v). The protein-free supernatant, obtained by speed centrifugation at 1,000 g, 4°C for 10 min, was collected for 2,3-BPG determination. A portion of the incubated red cell suspension was measured for pO_2 with a blood gas analyzer.

2.12.6 The effects of pH, temperature, A23187 and pO_2 on the quantity of deoxyhemoglobin.

The procedure was as follow:

1. **The effect of acidic pH on the quantity of deoxyhemoglobin.**
   - 1.1 The packed red cells were resuspended with HBSS pH ranging from 6.4 to 7.4 at approximately 5% hematocrit.
   - 1.2 The red cell suspensions were filled in a screw capped glass tube and then placed in 37°C water bath for 15 minutes.
   - 1.3 Removed all tubes from the water bath and immediately pipetted 0.1 ml of incubated red cell suspension into cuvette containing 0.9 ml of distilled water.
   - 1.4 The hemolyzed red cells were subject to quantitative estimation of deoxyhemoglobin by spectrometric method (Spectrophotometer, Model UV-2410PC, Shimadzu: Japan). The absorption spectra were scanned at the wavelength between 300 and 700 nm.

2. The effect of an elevated temperature on the quantity of deoxyhemoglobin.

2.1 The packed red cells were resuspended with HBSS pH 7.4 at approximately 5% hematocrit.

2.2 The red cell suspensions were filled in a screw capped glass tube and then placed to incubate at different temperature (35°C, 37°C, 39°C, 41°C, 43°C and 45°C) for 15 minutes.

2.3 The tubes were removed from the water bath. After that, 0.1 ml of each incubated red cell suspension was pipetted into cuvette containing 0.9 ml of distilled water.

2.4 The absorption spectrums were scanned by spectrophotometer at the wavelength between 300 and 700 nm.

3. The effect of A 23187 on the quantity of deoxyhemoglobin.

3.1 The packed red cells were resuspended with HBSS pH 7.4 at approximately 5% hematocrit.

3.2 Pipetted 0.5 ml of red cell suspension into a screw capped tube that containing 1.25 µl, 2.5 µl, 3.75 µl, 5 µl or 7.5 µl of 1.00 mM A23187 and not containing A23187 to serve as control. All tubes were gently mixed and incubated in a 37°C water bath for 15 minutes.

3.3 Removed all tubes from water bath and immediately pipetted 0.1 ml of incubated red cell suspension into 0.9 ml of distilled water.

3.4 The absorption spectrums were scanned by spectrophotometer at the wavelength between 300 and 700 nm.

4. The effect of a marked decrease in pO_2 on the quantity of deoxyhemoglobin.

4.1 The packed red cells were resuspended with HBSS pH 7.4 at approximately 5% hematocrit.

4.2 The red cell suspensions were filled in a screw capped glass tube. Each tube was blown with nitrogen gas for 5-15 minutes and not blown with nitogen gas to serve as control and then sealed with a cap.

4.3 All tubes were placed in a 37°C water bath for 15 minutes.

4.4 Removed all tubes from water bath and immediately, pipetted 0.1 ml of incubated red cell suspension into 0.9 ml of distilled water.

4.5 The absorption spectrums were scanned by spectrophotometer at the wavelength between 300 and 700 nm.

2.13 Statistical analysis

The results of erythrocyte 2,3-BPG levels and all of the parameters were expressed as mean ± SD. The 5% level of significance was selected to analysis of experimental data.

2.13.1 The effects of anticoagulants on erythrocyte 2,3-BPG levels in fresh blood and stored blood at 4°C for 5 days.

In each type of anticoagulants, the levels of 2,3-BPG obtained from fresh blood were compared to storage values using the student *paired t*-test for two means.

2.13.2 The effects of storage on erythrocyte 2,3-BPG levels when potassium oxalate was used as anticoagulant.

At different times of storage, the levels of erythrocyte 2,3-BPG were compared to fresh blood value using the student *paired t*-test for two means.

2.13.3 The effects of exercise program on carbohydrate and lipid metabolism.

The results of erythrocyte 2,3-BPG levels, lactate, triglycerides and glucose levels obtained from each of exercise programs were compared to sedentary control value using the one way analysis of variance (one-way ANOVA) for multiple means.