



001220

INTRAERYTHROCYTIC CHANGES OF METABOLIC
REGULATOR, FRUCTOSE 1,6 DIPHOSPHATE (FDP) IN VITRO

BY

SUPEECHA SIRIWAROOTHAKULE

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
(PHARMACOLOGY)

IN THE
FACULTY OF GRADUATE STUDIES
OF

MAHIDOL UNIVERSITY

1986

อภินันทนาการ

จาก

Faculty of Graduate Studies

This thesis

entitled

INTRAEERYTHROCYTIC CHANGES OF METABOLIC
REGULATOR, FRUCTOSE 1,6 DIPHOSPHATE (FDP) IN VITRO

was submitted to the Faculty of Graduate Studies, Mahidol
University for the Master of Science degree on

May 2nd, 1985

Supeecha Siriwarothakule

Supeecha Siriwarothakule

Candidate

Udom Chantharaksri

Udom Chantharaksri, Ph.D.

Preceptor

Suthat Fucharoen

Suthat Fucharoen, M.D.

Co-preceptor

Pairote Prempree

Pairote Prempree, Ph.D.

Monthree Chulasamaya

Monthree Chulasamaya, M.D., Ph.D.

Dean

Faculty of Graduate Studies

Mahidol University

Dean

Faculty of Science

Mahidol University

EVALUATION OF THE FINAL EXAMINATION
THE DEFENSE OF THESIS

.....

We, the members of the Supervisory Graduate Committee

for

SUPEECHA SIRIWAROTHAKULE

unanimously approve the thesis entitled

INTRAEERYTHROCYTIC CHANGES OF METABOLIC
REGULATOR, FRUCTOSE 1,6 DIPHOSPHATE (FDP) IN VITRO

We further agree that she has satisfactorily defended her thesis
at the examination given by the Supervisory Committee
on

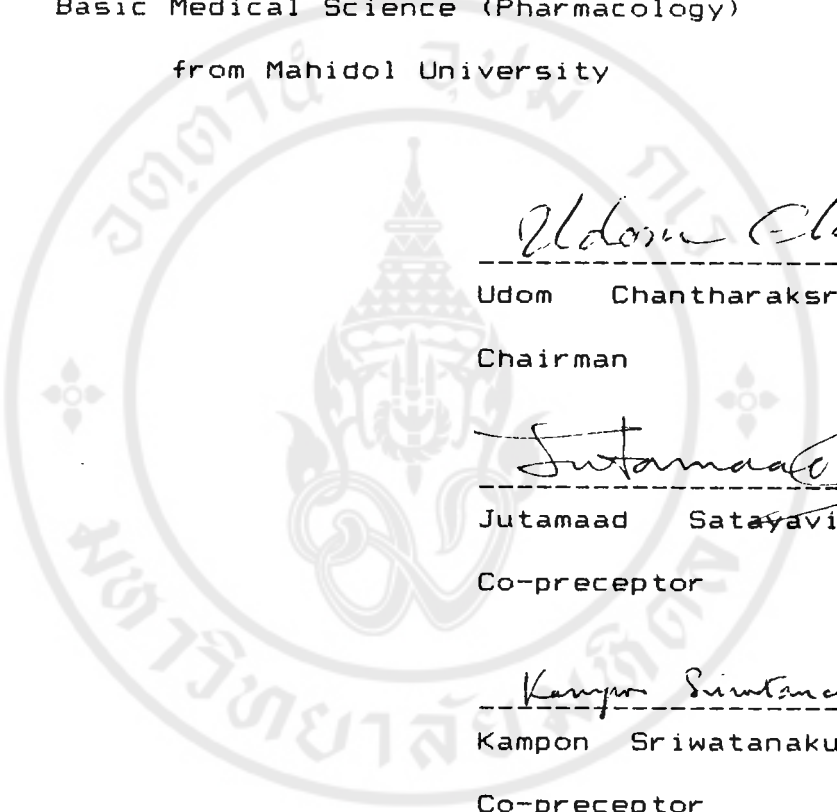
May 2nd, 1986

Copyright by Mahidol University

We recommend therefore that

SUPEECHA SIRIWARDTHAKULE

be awarded the degree of Master of Science in
Basic Medical Science (Pharmacology)
from Mahidol University



Udom Chantharaksri

Udom Chantharaksri, Ph.D.

Chairman

Jutamaad Satayavivad

Jutamaad Satayavivad, Ph.D.

Co-preceptor

Kampon Sriwatanakul

Kampon Sriwatanakul, M.D., Ph.D.

Co-preceptor

M. Chulasamaya

Monthree Chulasamaya, M.D., Ph.D.

Dean

Faculty of Graduate Studies

Mahidol University

Kampon Sriwatanakul

Kampon Sriwatanakul, M.D., Ph.D.

Chairman

Department of Pharmacology

Faculty of Science,

Mahidol University

Copyright by Mahidol University

BIOGRAPHY

Name : SUPEECHA SIRIWAROTHAKULE

Date of Birth : December 20th , 1959

Place of Birth : Bangkok , Thailand

Institutions Attended :

Mathayom Satit Srinakarinwiroj Prasarnmitr School,
Bangkok

March, 1975 Certificate of Mathayom Suksa III

Mathayom Satit Srinakarinwiroj Prasarnmitr School,
Bangkok

March, 1977 Certificate of Mathayom Suksa V

Faculty of Pharmaceutical Science, Chulalongkorn
University, Bangkok

March, 1982 Bachelor of Science in Pharmacy

Copyright by Mahidol University

ACKNOWLEDGMENTS

I would like to express my deep gratitude and appreciation to my advisor, Dr. Udom Chantharaksri, for his valuable guidance, suggestion, enthusiasm and kindness throughout this study.

I am very grateful to Dr. Jutamaad Satayavivad and Dr. Kampon Sriwatanakul, the advisory committee, for their valuable suggestion and criticism of this thesis.

My deep appreciation is expressed to Dr. Suthat Fucharoen, the Thalassemia center, Division of Haematology, Siriraj Hospital and Dr. Gobchai Puavilai, Department of Medicine, Ramathibodi hospital for their encouragement and providing the blood samples. My appreciation is also extended to Dr. Pimol Chiewsilp, Director Blood Bank, Department of Pathology, Ramathibodi Hospital for her allowance to obtain normal human blood of volunteers. And I would like to thank to the staffs of Blood Bank for their helpfulness to collect normal blood samples.

I am also grateful to Dr. Wittaya Tonsuwannont for his valuable suggestion in this studies and to Miss Varawadee Nerdnoi for her help in typing this thesis. My gratitude are also extended to Mr. Warakom Nirdnoy and Mr. Dejchai Siriwarothakule for their help on preparing this thesis.

Finally, I wish to express my appreciation to all other staff members, my friends and my parent for their encouragement and kindness.

Copyright by Mahidol University

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGMENTS	v1
LIST OF FIGURES	1x
LIST OF TABLES	x
LIST OF ABBREVIATIONS	x1
ABSTRACT	1
INTRODUCTION	3
OBJECTIVES OF RESEARCH	30
MATERIALS AND METHODS	
A. Animals and Human Subjects	31
B. Chemicals	32
C. Preparation of reagents	33
C.1 ACD, CPD, SALINE, PBS, PBS-D	33
C.2 Standard FDP	33
C.3 Reagents for the Assay of FDP	34
D. Collections and Preparations of Blood Samples	35
D.1 Animals	35
D.2 Human	36
E. Determination of FDP	38
E.1 Principle	38
E.2 Assay Protocol	39
E.3 Calculation	43

Copyright by Mahidol University

TABLE OF CONTENTS (CONT.)

	PAGE
RESULTS	
A. Effect of Citrate and Buffers on	
Intraerythrocytic FDP	44
A.1 Effects of pH in Citrate Buffer	49
A.2 Comparative Effects of ACD and Heparin	49
B. Levels of Intraerythrocytic FDP in Health	
and Diseases	
B.1 Fresh Blood from Volunteers	56
B.2 Blood of Diabetic Patients	56
B.3 Thalassemia	
-Nonsplenectomized	57
-Splenectomized	57
DISCUSSION	64
REFERENCES	69

Copyright by Mahidol University

LIST OF FIGURES

FIGURE		PAGE
1.	Glycolytic Pathway, Hexose Monophosphate Shunt and 2,3-DPG Branching Pathway in the Erythrocyte	6
2.	The Normal Oxygen Dissociation Curve	10
3.	Structural Formulae of Fructose 1,6 Diphosphate	22
4.	Standard Curve of Fructose 1,6 diphosphate	41
5.	Effect of ACD on the levels of FDP in Rat RBC	46
6.	Effect of CPD on the levels of FDP in Rat RBC	47
7.	Effect of PBS-D on FDP level in Rat RBC	48
8.	Effect of Citrate and pH on FDP level in Rat RBC	51
9.	Percent Decrease of FDP in various pH of Citrate	52
10.	Effect of Anticoagulant (Heparin or ACD) on Human RBC	54
11.	Effect of PBS-D on FDP levels in Human RBC	55
12.	Levels of FDP in Normal and Patients Human RBC Before and After incubated with PBS-D	63

LIST OF TABLES

TABLE		PAGE
1.	Enzymes Sensitive to Regulation by Citrate	26
2.	Allosteric Modifiers of Mammalian Phosphofructokinase	27
3.	Concentration of Fructose 1,6 Diphosphate in RBC in the presence of Different Incubating Buffers	45
4.	FDP Levels in Packed RBC after Incubate with Various pH Values of Citrate and PBS, and ACD for 1 hr	50
5.	Effect of pH in Citrat Buffer on the Levels of FDP	50
6.	Levels of FDP in RBC of Normal Volunteers after Incubated in vitro at 10 °C with PBS-D for 1 hr and 24 hr	59
7.	FDP levels of Diabetic Patients at Various Times of Incubated Period in the Presence of PBS-D	60
8.	Levels of FDP in RBC of Thalassemic Nonsplenectomized Patients after Incubated in vitro at 10 °C with PBS-D for 1 hr and 24 hr....	61
9.	FDP levels of Thalassemic Splenectomized Patients at Various Time of Incubated Period in the Presence of PBS-D	62

Copyright by Mahidol University

LIST OF ABBREVIATIONS

ACD	Acid Citrate Dextrose
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CPD	Citrate Phosphate Dextrose
°C	Degree celcius
dL	Deciliter
DM	Diabetes Mellitus
2,3 DPG	2,3 Diphosphoglycerate
F6P	Fructose 6 Phosphate
FDP	Fructose 1,6 Diphosphate
GAPD-LDH	Glyceraldehyde Phosphate Dehydrogenase - Lactate Dehydrogenase
GDH/TIM	Glycerol - 3 - phosphate Dehydrogenase / Triosephosphate Isomerase
gm	gram
Hb	Hemoglobin
HK	Hexokinase
hr	Hours
HMP	Hexose Monophosphate Shunt
i.u.	International Unit
M	Molar
mg	Milligram
mM	Millimolar
ml	Milliliter

min	Minutes
NS	Nonsplenectomized
μg	Microgram
μl	Microliter
μm	Micrometer
μmole	Micromolar
Pak-RBC	Packed Red Blood Cell
PBS	Phosphate Buffer Saline Solution
PBS-D	Phosphate Buffer Saline Dextrose Solution
PFK	Phosphofructokinase
PGF2α	Prostaglandin F2α
RBC	Red Blood Cell
SP	Splenectomized
Sp.gr.	Specific gravity
s/v	Surface area / Volume
Thal	Thalassemia
u	Units
w/w	Weight by Weight

Copyright by Mahidol University

ABSTRACT

Fructose 1,6 diphosphate (FDP), a metabolic regulator of the glycolytic pathway in the red blood cells is known to have the important roles in regulating cellular function and metabolism. Intraerythrocytic changes of FDP, subjected to various environmental conditions were followed in vitro, using a modified enzymatic method of Michal and Beutler (1974).

Both human and rat blood samples were taken into the heparinized plastic tubes, plasma was removed by centrifugation and red cells were washed with isotonic phosphate buffer before incubated at 10°C in different mediums for 1 and 24 hours. Packed red cells were deproteinized with perchloric acid, followed by K_2CO_3 neutralization before the FDP determinations.

At physiological pH (7.4), the presence of glucose significantly elevated intraerythrocytic levels of FDP after incubated for 24 hours. In the contrary, citrate which has been used as anticoagulant in clinical blood sampling and storage was found to lower the levels of FDP when incubated with the red cells. The suppressive effect of citrate on intraerythrocytic FDP was pH dependent; i.e. acidic citrate (ACD, pH 4.8) was more effective in lowering the level of FDP than the less acidic citrate. The change in pH per se, as varying the pH (from 7.4, 6.4, 5.5 to 4.8) in the phosphate buffer, used for red cell incubation caused no change to intraerythrocytic FDP under this in-

cubating condition.

The levels of intraerythrocytic FDP in the Diabetic (DM) and Thalassemia (THAL) patients were variably changed presumably by the pathological states. These could be due to prior exposure of red cells to relatively high blood glucose in the diabetic patients thus the intraerythrocytic levels of FDP were significantly elevated before the in vitro incubation, and then followed the pattern of FDP levels in normal blood from healthy volunteers, after incubated with the assigned medium for both 1 and 24 hours. This indicates that the glycolytic regulation of the DM's red cell is not defective. Levels of intraerythrocytic FDP in thalassemia were similarly elevated at pre- and post-1 hour incubations, while the levels of intraerythrocytic FDP after 24 hour-incubation of the splenectomized (SP) and non-splenectomized (NS) were in contrast difference. The FDP level of the SP-THAL dropped down below but that of the NP-THAL continuously elevated beyond their respective pre-incubation levels suggesting some glycolytic regulatory defects in the thalassemic red cells, of these SP-Thal in particular. The elevated FDP levels in THAL; of both NS and SP patients at pre- and 1 hour after post-incubation may be resulted from the underlining hyperglycemia in these patients. The defects in the glycolytic regulation in the splenectomized thalassemic red cells is obviously different from the non-splenectomized patients. The distinction in such defects merits further studies.

INTRODUCTION

Literature Reviews

Mature mammalian erythrocyte is one of the most specialized cells; lacking cytoplasmic organelles, i.e. nucleus, mitochondria and ribosomes (Winthrope, Lee, Boggs, Bithell, Foerster, Athens and Lukens, 1981). It is developed from erythroblast, the precursor nucleated red cell, resided in the bone marrow. Pronormoblast, basophilic normoblast, polychromatic normoblast and orthochromatic normoblast are the four consecutive developmental stages of nucleated erythroblasts before the nucleus is lost on its the reticulocyte young red cell stage, and eventually turning into mature red blood cell. In healthy adult, reticulocytes represents some 0.5-1.5 % of the circulating red cells. The abnormal rise in reticulocyte count is found in some diseases like sickle cell anemia and thalassemia. In thalassemia trait, reticulocyte count increased about 2-10 % (Wallach, 1974). The mature red cell is unable to synthesized new proteins, carry out the oxidative reactions associated with mitochondria nor undergo mitosis. It consists of little more than a membrane surrounding a solution of protein and electrolytes. More than 95 % of the protein is hemoglobin, the oxygen-transport protein. The remainders include those enzymes required for enzyme production and for the maintenance of hemoglobin in the functionally,

reduced state (Winthrobe, et al., 1974). The resting shape of normal human erythrocyte is that of a flattened, bilaterally indented structure, a shape often referred to as a "biconcave disc". The disc shape is well suited to erythrocyte function. The normal mean diameter of red cells, measured after drying and staining, is variously given as 7.2 to 7.9 microns. The surface area of the erythrocyte is about 140 microns. The ratio of s/v (surface area/volume) approaches the maximum possible value in such a shape, thereby facilitating gas transfer. Furthermore, the biconcave disc is more deformable than a sphere and can therefore undergo the necessary shape change for optimal movement within the microvasculature, (Besis, Lessin, Beutler, 1983; Winthrobe et al., 1981). Most red cells are destroyed by phagocytosis, many of them in the spleen. Red cell destruction by the spleen requires preliminary changes that result, among other things, in a lack of deformability. This may result from stiffening of the cytoplasm, as in sickle cell anemia or Heinz body anemia, from loss of red cell surface membrane, or from rigidity of membrane protein (Weed, 1970).

Hemoglobin (Hb) is responsible for binding, transport and delivery O_2 in the red cell in a remarkably efficient manner. The erythrocytic hemoglobin must be maintained in the reduced state (divalent form of the iron of Hb) required energy from anaerobic breakdown of glucose to pyruvic or lactic acid in the

Embden-Meyerhoff pathway (Fig.1). Unlike most other cells, the red cell lacks a citric acid cycle. The glycolytic energy gets red cell to perform its function efficiently, and survive in the circulation for its full life span of approximately 120 days. It is also needed for maintaining high levels of cellular potassium and low in calcium and sodium within the cell against a gradient imposed by the high plasma calcium and sodium and low plasma potassium levels, the sulfhydryl groups of red cell enzymes and of hemoglobin in the active, reduced form and to maintain the biconcave shape of the cell. If it is deprived of energy, the red cell shape changes from a biconcave disc to a sphere; sodium-lagged and potassium depleted. Red cells would gradually turn brown as hemoglobin is oxidized to methemoglobin by the very low concentration of O_2 . The cell would then be unable to perform its function on O_2 and CO_2 transport. Plasma glucose is its chief energy source. The red cell has the capacity to utilize several other substrates such as adenosine, inosine, fructose, mannose, galactose, lactate or dihydroxyacetone (Beutler, 1984; Beutler, 1983).

Anaerobic breakdown of glucose in the Embden-Meyerhoff pathway can result in phosphorylation of ADP to ATP, reduction of NAD to NADH and production of 2,3-diphosphoglycerate (2,3 DPG) which is a regulator of the oxygen affinity to hemoglobin. After formation of 1,3-diphosphoglyceric acid (1,3-DPG) the Embden-

GLYCOLYSIS IN THE ERYTHROCYTE

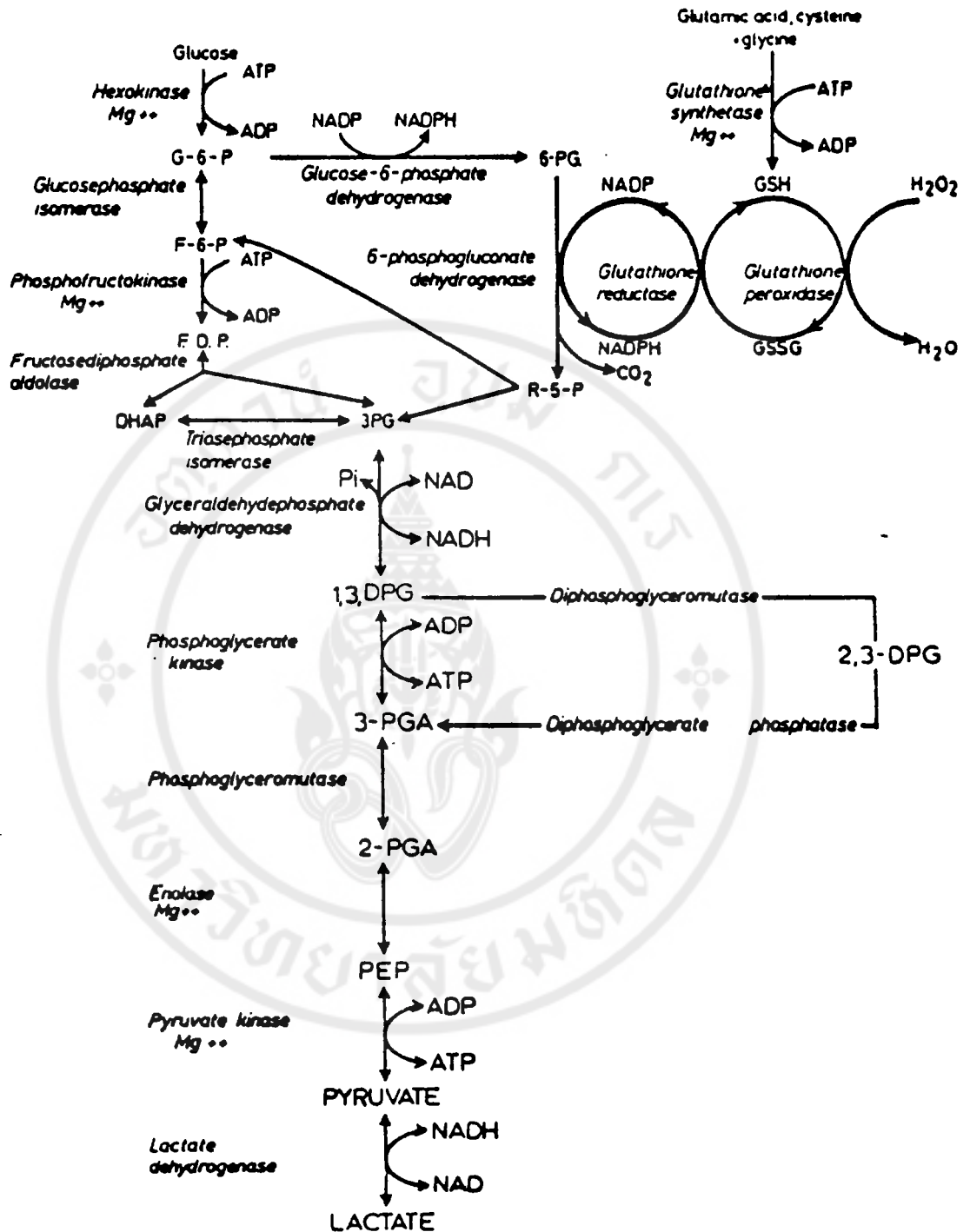


Fig. 1. Main glycolytic pathway and hexose monophosphate shunt with dependent glutathione system. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DHAP, dihydroxy acetone phosphate; 1,3 DPG, 1,3-Diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; FDP, fructose diphosphate; F-6-P, fructose-6-phosphate; H₂O₂, hydrogen peroxide; NAD, nicotinamide-adenine dinucleotide; NADP, nicotinamide-adenine dinucleotide phosphate; PEP, phosphoenol pyruvate; 3-PG, glyceraldehyde-3-phosphate; 6-PG, 6-phosphogluconate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; Pi, phosphate; R-5-P, ribulose-5-phosphate.

(From Williams, 1983, in Hematology)

Meyerhoff pathway branches, 1,3 DPG can be inverted to 3-phosphoglyceric acid directly through the phosphoglycerate kinase, resulting in the phosphorylation of ADP to ATP. Alternatively, it can be converted through the action of diphosphoglycerate mutase into 2,3 DPG, which in turn, can be catabolized to 3-phosphoglyceric acid through the action of a phosphatase (Beutler, 1984). The rates of all the metabolic processes of the erythrocyte depend on the nature of enzyme, number of enzyme molecules present, temperature, and concentrations of substrates, cofactors, activators, inhibitors and hydrogen ions within the cells (Beutler, 1983). An additional pathway of glucose metabolism in the red cell also exists in a direct oxidative shunt (Hexose monophosphate shunt). In this pathway the reduced coenzyme is the NADP instead of the NAD as in the Embden-Meyerhoff pathway. The principle function of reduced NADP (NADPH) appears to keep glutathione in the reduced state. Glutathione in the red cells maintains the sulfhydryl groups in the active reduced state (Beutler, 1983). Before glucose can be metabolized by the red cell, it must pass through the membrane. The transport of glucose into red cells is independent of insulin. The metabolism of glucose in red cell gives a net yield of 2 mole of ATP (Beutler, 1983).

The rate of glucose utilization is limited largely by the hexokinase (HK) and phosphofructokinase (PFK) reaction. Both

enzymes catalyze the reactions at relatively high pH optimum. Red cell glycolysis is pH sensitive; being stimulated by a rise in the pH. However, at higher pH than that of physiologic levels, the stimulation of hexokinase and phosphofructokinase activities merely results in the accumulation of fructose 1,6 diphosphate (FDP) and triosephosphates (Beutler, 1983). Younger red cells contain much higher levels of hexokinase than do the older ones (Brewer and Powell, 1963 ; Chapman and Schaumburg, 1967; Valentine, Oski, Paglia, Baughan, Schneider and Naiman, 1967). The enzyme is strongly inhibited by its end product, glucose-6-phosphate, and the inorganic phosphate ion has been reported to release this inhibition (Rose, Warms and O'Connell, 1964). The stimulatory effect of inorganic phosphate on the rate of glucose utilization by red cells is indeed not exerted through the hexokinase, but rather through stimulation of the phosphofructokinase reaction, resulting in lowering the glucose-6-phosphate concentration within the cell and thus hexokinase inhibition is released (Beutler, 1983). PFK is the important rate limiting step in the phosphorylation of fructose-6-phosphate by ATP to fructose-1,6-diphosphate. It requires magnesium for activity and is stimulated by both ADP and inorganic phosphate. The inhibitory effect of ATP on PFK diminishes the affinity of this enzyme for other substrate such as F6P. This interplay will lessen the rate of glucose consumption when the concentration of ATP is raised above normal. Whenever the fall in ATP concentration occurs, the

release of PFK inhibition is evident and glycolysis is stimulated to replenish the ATP (Grimes, 1980). Pyruvate kinase (PK) is another kinase which are important in the regulation of the rate of glycolysis. Like PFK, PK is also inhibited by ATP in a manner competitive with its substrate PEP (phosphoenolpyruvate) and the role of PK is to regulate the flow of substrate for 2,3 DPG formation. PK is inhibited by 2,3 DPG and is activated by FDP. At low concentrations of PEP the enzyme activity is greatly increased by FDP (Kahn, Marie, Garreau and Sprengers, 1978; Grimes, 1980). Red cell PK is allosterically regulated by FDP which is the known most potent effector (Grimes, 1980).

2,3 DPG, an important regulator of O_2 affinity of hemoglobin, is presented in 5 to 7 mmole per litre of packed red cells. It binds competitively with O_2 to the hemoglobin which provide a useful mechanism in delivery of O_2 to body tissues (Bonaventura, C., Bonaventura, J., Amiconi, Tentori, Brunori and Antonini, 1975). In many mammalian species, lowering the affinity of O_2 to Hb occurs during the development from fetus to young adult with an abrupt increase in 2,3 DPG levels, shifting the oxygen saturation curves to the right (Westhead, Kiener, Carroll and Gikner, 1984; Ranney and Shorma, 1983). Concentration of 2,3 DPG in the red cells is regulated by the activity of pyruvate kinase not only by the bifunctional enzyme Diphosphoglyceromutase / Diphosphoglycerate phosphatase as anticipated .,

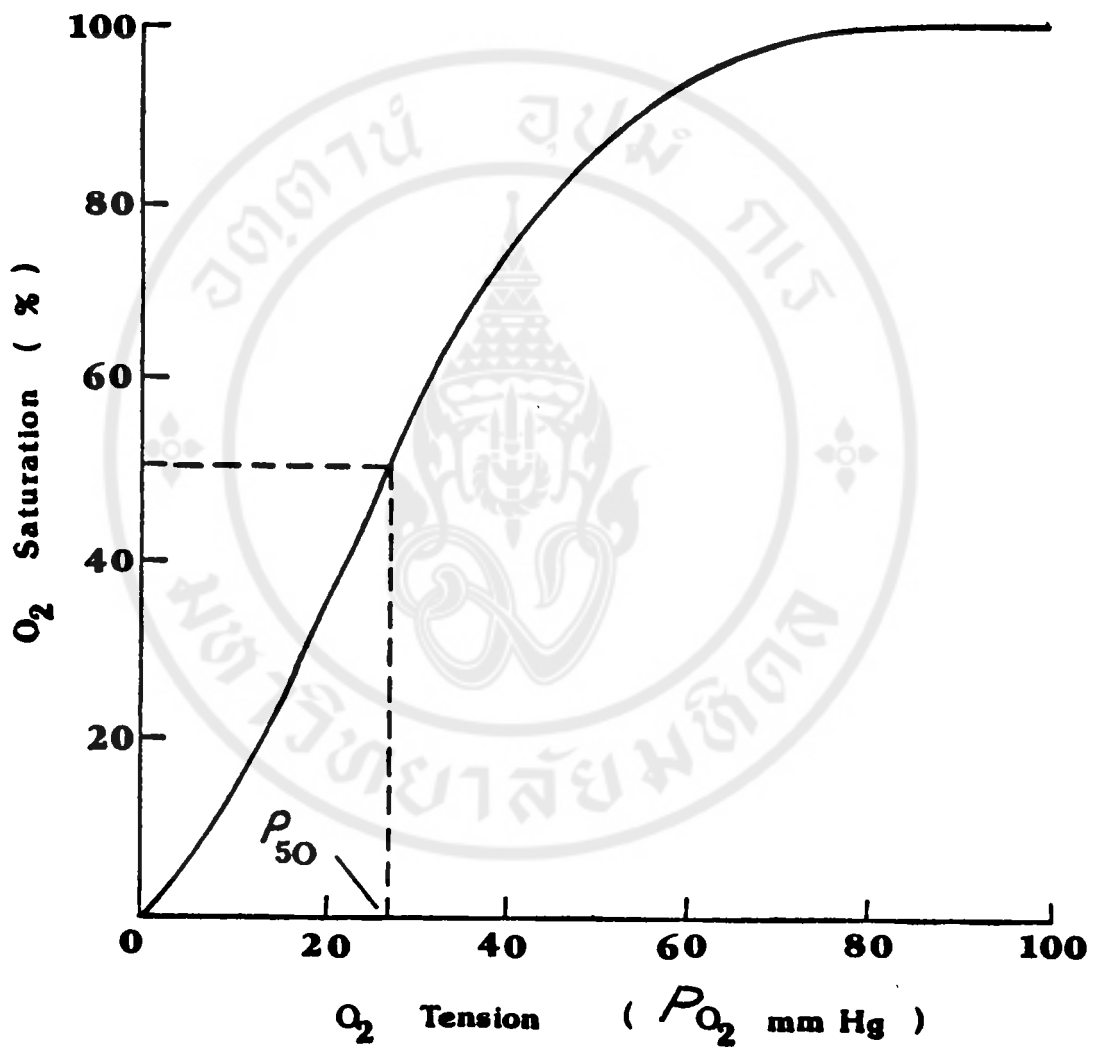


Fig 2 : The Normal Oxygen Dissociation Curve

(From Grimes, 1980, in Human Red Cell Metabolism)

Copyright by Mahidol University

(Grimes, 1980). Hydrogen ions inhibit the diphosphoglyceromutase reaction and stimulate the phosphatase reaction. The levels of red cell 2,3 DPG are exquisitely sensitive to pH; a rise in pH causes an elevation of 2,3 DPG levels, while acidosis results in 2,3 DPG depletion (Beutler, 1983). The fine control of pyruvate kinase activity is thus an essential mean in regulating the delivery of oxygen to body tissues in the erythrocytes (Westhead et al., 1984).

Both ATP and 2,3 DPG are essential components for the properly functioning red blood cells. ATP induces shape changes in human erythrocytes membranes; the phosphorylation by ATP on spectrin component of the erythrocyte membrane is known to occur (Birchmeier and Singer, 1977). Sheetz and Singer (1977) reported that erythrocyte ghosts undergo ATP-dependent shape changes, from crenated to disk to biconcave shape. These changes are specific only for ATP but other nucleotides are ineffective. The ability to maintain the biconcave shape of the red cells depends solely on the level of ATP or the turnover of ATP (Nakao, Nakao, and Yamazoe, 1960). The cytoplasmic level of ATP is associated with the ability of the cells to deform. Normal cellular deformability (the ability of the red cells to pass through a very narrow capillaries smaller than their own diameter) is the most important determinant of red cell lifespan in vivo (Weed, 1970). Red cell deformability reflects an essential property of membrane

fluidity to undergo rapid changes in shape in flowing through the microcirculation. Normal blood stored in ACD (Acid Citrate Dextrose) and CPD (Citrate Phosphate Dextrose) for 42 days undergoes increased in viscosity and decreased deformability. In parallel with this loss of deformability there is also a decline in the ATP and lipid contents of the cell as well as the s/v ratio (Grimes, 1980; Card, Mohands, Perkins and Shohet, 1982). The rate of deformability loss during storage increased with increased degrees of cell packing because of more rapid substrate depletion (Card et al., 1982). Within about 12 hr of glucose depletion, the red cell levels of both 2,3 DPG and ATP fall. The storage of blood under standard condition in ACD at 4°C gives rise to a progressive increase in the O₂ affinity; i.e., a leftwards shift of the O₂ dissociation curve. The shift is noticeable occurred with the fall in 2,3 DPG level during two weeks of blood storage (Grimes, 1980; Tanaka and Asakura, 1984). The major effect of blood preserving agents is to maintain the levels of ATP and 2,3 DPG at the normal prestorage values. The process is referred to as rejuvenation of the RBC.

Dextrose in ACD or CPD is supplied as substrate of the glycolytic pathway, inorganic phosphate added in CPD (a modified ACD) increases the buffering capacity and is isotonic; its other advantage is of a higher pH than ACD at which preservation of 2,3 DPG is claimed to be better (Masouredis, 1983). The designated

Copyright by Mahidol University

conditions for preserving erythrocytes are yet optimal for the anticipated risk to maintain the various important components blood. Standard preservative medium at 4°C results in a deterioration of the red cells. Blood preservatives composed of ACD and CPD have been supplemented with adenine to extend the present 21 days shelf life to 35 or 42 days (Messeter, Ugandder, Monti, Lundh and Low, 1977). Adenine entered the red blood cell, is incorporated eventually into ATP which is required for phosphorylation of glucose, the first step in the energy yielding glycolytic pathways which are responsible for maintaining the viable red cells. However, adenine has no influence on the levels of red cell 2,3 DPG. 2,3 DPG levels are significantly better maintained in CPD-adenine preservatives that contained 15 mM concentrations of inosine. Inosine is of known value in maintaining 2,3 DPG for prolonged (5-6 weeks) liquid storage (Dawson, 1977). Other supplement added in the storage of the red blood cells is guanine nucleotide. Addition of guanosine to the storage medium improves the quality of the erythrocytes (de Verdier, Strauss, Ericson, Akerblom and Hogman, 1981) expressed as post transfusion viability, and concentrations of purine nucleoside triphosphate and 2,3 DPG. The rapid incorporation of guanosine into guanine nucleotides of erythrocytes is evident in the work of Brown and Parks (1973), Kong and Parks (1975), and Henderson et al., (1974,1978). The synthesis of guanine nucleotides could be used as a practical procedure for rejuvenation of stored erythrocytes. It has

been suggested that GTP has a less pronounced inhibiting effect than ATP on the binding of fructose- 6-phosphate to the active center of phosphofructokinase (De Verdier et al.,1981) and thus on glycolysis in general (Ericson, Niklasson and de Verdier, 1985).

The erythrocytic level of 2,3 DPG has been reported to fall with age from a mean value of 14.9 umole/g Hemoglobin (ages of 18 to 24 yrs) to 13.9 umole/g hemoglobin (at age 75 to 84 yrs) (Purcell and Borzovic, 1974). This decrease is statistically significant. It is possible that the decrease of 2,3 DPG in the red cell, is related to the lower concentrations of plasma inorganic phosphate found in old age. Increased concentrations of 2,3 DPG and plasma inorganic phosphate were found in children between 1 and 12 year of age (Card and Brain, 1973). The osmotic fragility is increased in older individuals in comparison with younger subjects (Detraglia, Cook, Stasiw, and Cerny, 1974; Araki and Rifkind, 1980). This phenomenon may be related to the increased MCV (mean corpuscular volume) and decreased MCHC (mean corpuscular hemoglobin concentration) of the red cells in older people (Araki and Rifkind, 1980). The rate of hemolysis, in contrast, is more rapid in older individuals, perhaps due to changes in the erythrocyte membrane (Araki and Rifkind, 1980). The red cells of patients with anemia generate increased amounts of 2,3 DPG (Pollock and Cotter, 1973; Bellingham and Grimes,

1973) and this compound has the capacity to combine with deoxygenated hemoglobin and decrease its affinity for oxygen (Torrance, Jacobs, Lenfant and Finch, 1969). In some anemic subjects over 65 years old, the increase of 2,3 DPG was less than expected for the given degree of anemia. In some elderly subjects decreased red cell 2,3 DPG may impair the mechanism of adaptation to hypoxia or anemia (Purcell and Brozovic, 1974). We have known that the life span of red cell is about 120 days. The glycolytic enzyme activity in older red cells are probably declined which leads to membrane alterations and change in some of the overall properties of the cell such that its deformability, mechanical function and osmotic fragility are all decreased. Almost all the enzymes show a diminished activity in older cells. A decline in the levels of 2,3 DPG and the activity of the HMP (hexose monophosphate shunt) occurs with aging of red cells (Grimes, 1980).

In anemic patients, the hemoglobin levels are low (6-12 /dl) with an accompanying right shift of the O_2 -dissociation curve to the right, indicating a decrease in oxygen affinity. Each fall of 1 g in the hemoglobin level will result in a rise in 2,3 DPG of 0.23 mM (Torrance et al., 1970). In certain of the anemias there will be specific factors present which stimulate 2,3 DPG synthesis; for example the acid-base imbalance, or the rise of plasma inorganic phosphate as in renal disease certain cases

of anemias, resulting from congenital block in glycolysis shows pyruvate kinase deficient, but with an elevated red cell levels of 2,3 DPG because the 2,3 DPG formation precedes PK, the rate-limiting step. In some other hexokinase deficiencies, the formation of 2,3 DPG is less resulting from the glycolytic blockade (Grimes, 1980). They are likely caused by defects in cellular metabolism including glucose-6-phosphate dehydrogenase deficiency. Deficiency of PFK may be associated with a mild form of hemolytic anemia has been reported by Waterberry and Frenkel (1972). Most of the anemias due to intrinsic abnormalities are caused by heredity defects in the formation or protection of the cellular constituents of circulating red cells. Such defects may lead to abnormalities in 1) red cell membrane, 2) red cell enzymes 3) globins, and 4) heme. Most of the hemolytic anemia (increase of red cell destruction) can be assigned to one of these four abnormalities (Erslev, 1983). Life span of red cells in anemic patients is usually shorter than normal. Since normal red cells survive normally when injected into healthy recipients but have a shorter life span in anemia of chronic renal failure (uremia), it appears that the metabolic environment is the determining cause (Erslev, 1983). However, most red cell enzymes show normal or increased activity in uremia, and the intracellular level of ATP is higher (Mansell and Grimes, 1979). Only the levels of transketolase active in the HMP shunt, and ATPase, powering of the $\text{Na}^+ - \text{K}^+$ membrane pumps, are decreased. The

decreased activity of the $\text{Na}^+ - \text{K}^+$ pumps could also cause changes in red cell shape and rigidity and in turn the life span of red cells (Erslev, 1983). Hereditary spherocytosis (HS), or congenital hemolytic jaundice shows clearly increased in osmotic fragility of the red blood cells accompanying an intrinsic metabolic abnormality in HS erythrocytes, and a decrease in S/V ratio. These defects may lead to their trapping and eventual destruction in the spleen and autohemolysis. Increases of glycolysis sodium flux and sodium-potassium ATPase activity play important role in the abnormality of HS red cells. Accelerated glycolysis purposes to compensate for an abnormality of cell membrane permeability is the usual accompanying consequence when glucose is limited the hypermetabolism would lead to a rapid and premature decline in ATP and 2,3 DPG, volume control is then lost and autohemolysis occurred (Jande and Cooper, 1983). For sickle cell disease the RBC shows abnormal shape and loss of deformability. Sickle cells and spherocytes are poorly deformable and thus become easily trapped in microcirculation (Marion, Henry and Lusher, 1974).

Both functional and metabolic abnormalities are evidently occurred to the red cells of heterozygous β thalassemia. Probably, the best known is the decrease in osmotic fragility especially after a sterile incubation in vitro for 24 hr (Weatherall and Clegg, 1981; Gunn, Silvers and Rosse, 1972).

The addition of glucose during in vitro incubation prevents the β -thalassemic cells to become more resistant to osmotic lysis. There is a marked increased outflux of K^+ from thalassemic red cells with a minimal increase of intracellular Na^+ during incubation; almost no compensatory increase in Na^+ occurs. The cells lose ATP more rapidly than normal red cells. If glucose is added to the incubation medium the Na^+-K^+ pump can be activated to counteract the excessive loss of K^+ and water from the cell (Knox-Macaulay and Weatherall, 1974; Weatherall and Clegg, 1981). This change is due to an increase in selective permeability of the membrane to K^+ with regard to abnormality of the membrane function (Gunn et al., 1972). The resistance to osmotic lysis has been generally attributed to changes in cell shape to the concomitant loss of K^+ and water and the shrinkage of red cells. RBC of thalassemic major patients have lost considerable amounts intracellular K^+ , and decrease in size (Gunn et al., 1972). The intracellular calcium (Ca^{2+}) concentration in RBC is controlled by a slow passive influx through a relatively impermeable membrane and by active efflux, catalyzed by Ca^{2+} , Mg^{2+} -ATPase. Precipitation of globin chains in thalassemic RBC may interfere with many aspects of membrane function. One major defect in the membrane of the thalassemic RBC leads to increased intraerythrocyte Ca^{2+} content which seems to be more pronounced in splenectomized patients (Shalev, Mogilner, Shinar, Rachmilewitz and Schrier, 1984).

The increased viscosity of blood in diabetic patients is caused by a number of factors such as reduction of individual red cell deformability, the enhancement of red cell aggregation and an increase in plasma viscosity caused by abnormal plasma proteins. The decreased red cell deformability is largely independent of the onset, duration and complications, but depends critically on the abnormal incidence of metabolic control in the diabetic individuals (Schonbein and Volger, 1976). Red blood cells in diabetics are more adhesive to endothelial surfaces than those of non-diabetic and is directly related to vascular complications in the diabetics (Wautier, Paton, Wantier, Pintigny, Abadie, Passa and Caen, 1981). These suggest that there is an intrinsic erythrocyte abnormality, related to vascular disease in diabetic patients. The reduced deformability of red cells in diabetic is due to an elevation of intracellular hemoglobin A1C which could raise intraerythrocyte viscosity. Hb A1C, the most abundant minor Hb component in human erythrocyte, is formed by the condensation of glucose with the N-terminal amino groups of the β -chain of HbA. It is increased by two fold in diabetic patients (McMillan, Utterback, Pumo and Barbara, 1978; Bunn, Haney, Kamin, Gabbay and Gallop, 1976). Erythrocytic membrane fluidity is not different between DM and normal. It does not play a major role in the alterations of the physical properties of blood seen in type 1 DM (Hill and Court, 1983). The abnormal level of 2,3 DPG in

diabetic patient is related to the presence of vascular complication. Patient with vascular complication shows significantly elevated blood level of 2,3 DPG. Elevation of 2,3 DPG level may reflect the attempt of red blood cell to compensate for tissue hypoxia in diabetes with vascular disease (Kanter, Bessman and Bessaman, 1975). Increased erythrocytic 2,3 DPG is associated with increased hemoglobin glycosylation (Hb A1C) in patients with Type 1 DM who have no cause or symptom of hypoxic stress. It is the changes in position of the erythrocyte oxygen dissociation curve that modulates the increase in 2,3 DPG synthesis for hemoglobin A1C formation (Robert, Stony and Ryall, 1984). Flux of glucose through PPP (pentose phosphate pathway or hexose mono phosphate shunt) was decreased by 70% in diabetic rats heart is parallel with a suitable decreased flux through the glycolytic route because there is a decrease in hexokinase activity. Level of F-2,6P₂, was a regulator of PFK activity in the diabetic myocardium. There is a rise in Hexose monophosphate and fall in FDP level. Decrease in the content of certain form of hexokinase is a characteristic response of insulin dependent in diabetic patients, in the presence lactating mamary glands and adipose tissue. In contrast, the increase hexokinase activities is registered in tissues, freely permeable to glucose without the need of insulin for glucose uptake e.g., Kidney and Lens (Sochor, Gonzalez and Mclean, 1984). Hexokinase activity of lens increases two-fold in alloxan-diabetic rats four weeks after induction of

diabetes (Gonzalez, Sochor, Hothersall and Mclean, 1978). Madsen and Ditzel (1982) reported that levels 2,3DPG in red blood cell was significantly increased in the pregnant diabetic women, but the 2,3 DPG induced change in Hb-O₂ affinity was impaired and fetal hypoxia occurred.

Fructose 1,6 diphosphate (FDP) is one of the metabolic products in glycolytic pathway that plays a central role in the regulation of several pathways of cellular energy metabolism (Perroni et al., 1980). It stimulates the glucose catabolism via enzyme PEK and PK in the glycolytic pathway. (Kirtley and McKay, 1977) and potentiates ATP production. The increase of ATP levels after FDP administration had been shown in adipose tissue and human blood in vitro (Prosdocimi et al., 1979). In vivo studies of an oral glucose loading, FDP infusion increased blood ATP level significantly and glucose level was significantly lower when compared with fructose and saline infusion (Perroni et al., 1980). Prosdocimi, et al. (1979) reported that only FDP, not others (G1P, G6P, fructose, F1P and F6P) strongly potentiated the lipolysis in adipose tissue induced by noradrenaline and theophylline. Both increased in basal ATP and cAMP levels accumulation are good index for lipolysis in adipose tissue. They suggested that FDP stimulates glycolysis and consequently release ATP for the synthesis of cAMP. The activity of FDP on lipolysis does not caused by its hydrolysed products (Fructose + Pi). FDP inhibits

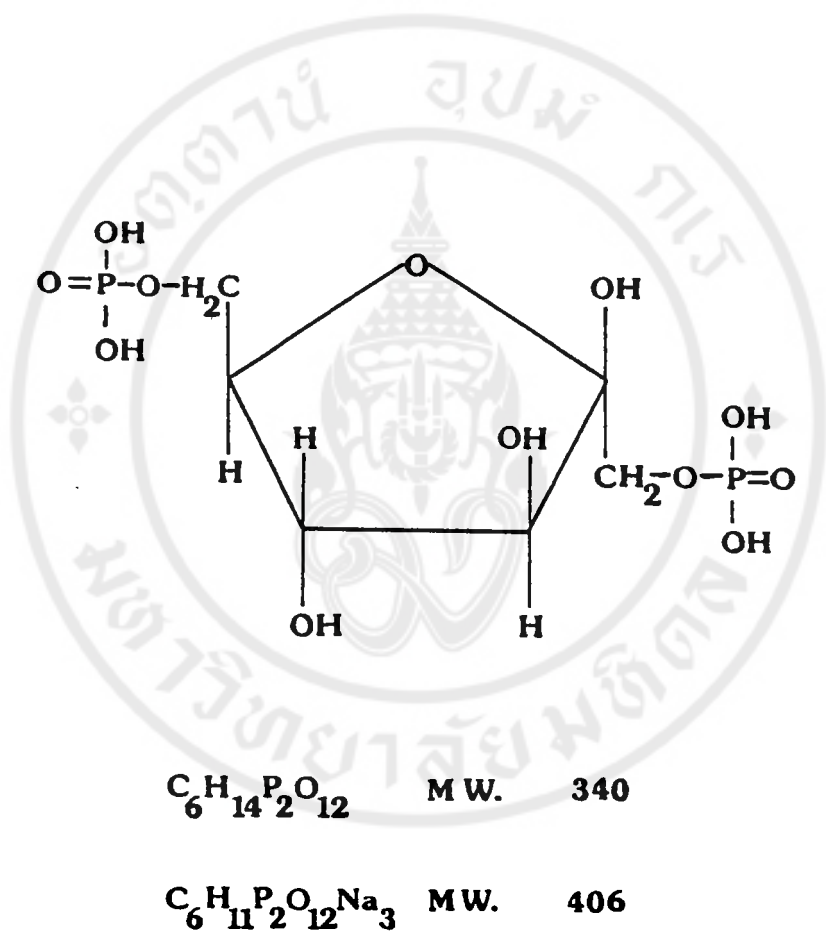


Fig 3 : Structural Formulae of Fructose 1,6 diphosphate (FDP)

Copyright by Mahidol University

gluconeogenesis, stimulated glycogen synthesis and inhibits glycogen breakdown while blocking utilization of glucose in pathways other than glycolysis (Kirtley and McKay, 1977). The regulating role in smooth muscle function (Costrini et al., 1980) of cyclic nucleotide suggested that FDP may modify smooth muscle response to stimuli such as uterine smooth muscle to $\text{PGF}_{2\alpha}$ and oxytocin. They found that FDP seems to be the only compound capable of modulating uterine smooth muscle responses to hormonal and pharmacological stimuli of $\text{PGF}_{2\alpha}$ and oxytocin. It amplifies the effect under condition of subliminal stimuli of these two compound. The increase in the energy availability may promote the onset of contractile responses and speed up. The anticontractile response under excessively or severe stimuli, indicating its action via the ionic movements. The pharmacological effects of FDP seem to be dependent upon the capability to induce changes of ionic permeability of cell membrane which in turn influences glucose metabolism and PFK activity in particular (Lazzarino et al., 1984).

In stored blood (ACD sol., 4°C) intraerythrocyte 2,3 DPG levels are reduced. A shift to the left of the oxyhemoglobin dissociation curve can then occur, with an enhanced Hb-O_2 affinity and impaired tissue oxygen delivery. After FDP infusion in polytransfused patients a rapid and consistent increase of 2,3 DPG levels occurred (Iapichino, Pesenti, Radrizzani, Solca and Vesconi, 1978). The erythrocytic deformability seems to be

increased. FDP has been used in clinical treatment of the many post-operative condition such as intestinal transit, adynamic ileus with combined in total parenteral nutrition(TPN). The addition of FDP to glucose and nutrients can reduce the intestinal recanalization times, the recovery of the bowel peristalsis, results in an energetic counteraction to the effects of catecholamines (Manani et al., 1977; Manani et al., 1982). FDP added in TPN results in good control of plasma glucose and acceptable plasma insulin concentrations, as well as in reduction of insulin administration. It promotes glucose utilization probably by an insulin independent mechanism (Giordano and Santo, 1983), decreasing blood glucose levels and increasing ATP and 2,3 DPG serum concentrations (Iapichino et al, 1978; Dionigi et al., 1978). FDP was also given to patients following trauma, surgery or severe anemia for improving erythrocyte function, favour tissue oxygenation, and erythrocyte resistance to hemolysis (Costrini et al., 1980; Iapichino, 1980; Dionigi et al., 1978). FDP was shown to be effective in improving peripheral ischemia and particularly in case of severe peripheral arterial troubles. The metabolism of erythrocytes in most of these patients is highly impaired. Concomitantly, the deformability of the cells decreases, hindered blood flow in the capillaries. The functionality of erythrocytes may worsen for ischemia such as in diabetic but sometimes (for instance in case of polycythemia and sickle cell anemia) it is the principle cause of ischemia. Activities of FDP indicate

the importance of rheologic variations in modifying ischemia (Marsilli, Pata and Saratti).

Endogenous metabolic product that play significant role in regulating cellular glucose metabolism is citrate. The metabolic function of citrate in glucose and fatty acid metabolism include 1) chelation of metal ions 2) generation of extramitochondrial acetyl-CoA for biosynthetic pathways 3)* regulation (activation or inhibition) of the enzymes (Nielsen (1983). Many enzymes are sensitive to regulation by citrate (Table 1). One of the earliest recognized and best confirmed regulatory steps is its inhibition on the key enzyme, PFK by an allosteric mechanism. Plasma citrate may reflect the cytosolic citrate regulation of intracellular glucose metabolism. Citrate may pass cell membrane as a monoanion, complex-bound to Ca^{2+} or Mg^{2+} , not a free anion form. One evidence that PFK is a regulatory glycolytic enzyme is its sensitivity to allosteric regulation. The interconversion via a phosphorylation-dephosphorylation mechanism in the form of polymer. PFK are allosterically influenced by a large number of inhibitors and activators (table 2). Among the strongest inhibitors are ATP and citrate while FDP, AMP, cAMP and G1,6P₂ are activators. Citrate has been found to inhibit PFK from many tissues in both animals and man. Citrate can potentiate the inhibitory effect of ATP; decrease the binding of the activators AMP, cAMP, ADP and F6P and inhibits the activation of PFK by FDP., (Torn-

Table 1. Enzymes sensitive to regulation by citrate.

Enzyme	Metabolic function	Effect of citrate	Animal and tissue origin of enzymes
Acetyl-CoA carboxylase* (EC 6.4.1.2)	fatty acid synthesis	activation	rat adipose tissue Chicken liver rat mammary gland
Citrate synthase ⁺ (EC 4.1.3.7)	citric acid cycle	inhibition	beef heart mouse liver
Fructose-1,6-diphosphatase* (EC 3.1.3.11)	gluconeogenesis thermogenesis	activation	rat skeletal muscle
Phosphofructokinase* (EC 2.7.1.11)	glycolysis	inhibition	rabbit skeletal muscle rat heart muscle sheep brain rat liver rat adipose tissue rat kidney cortex human adipose tissue pig spleen rat mammary gland rat thyroid
Phosphoglucomutase* (EC 2.7.5.1)	glycogenolysis glycogenesis	inhibition	mouse skeletal muscle rat adipose tissue
Pyruvate dehydrogenase ⁺ complex (EC 1.2.4.1)	decarboxylation of pyruvate to acetyl-CoA	inhibition	rat liver rat skeletal muscle rat adipose tissue

*) Cytosolic enzyme.+) Mitochondrial enzyme.

(From Neilsen, 1983, Vol. 30, Danish Medical Bulletin)

Copyright by Mahidol University

Table 2. Allosteric modifiers of mammalian phosphofructokinase.

Inhibitors	Activators
ATP [*]	Fructose-1,6-P ^{**}
Citrate	AMP ⁺
3-phosphoglycerate	cyclic AMP ⁺
2-phosphoglycerate	glucose-1,6-P ⁺
2,3-diphosphoglycerate	ADP ⁺
Phosphoenolpyruvate	Fructose-6-P ^{**}
	Pi ⁺
	NH ₄

*) both substrate/product and regulator of the reaction.

+) activator and deinhibitor counteracting inhibition by ATP and citrate.

(From Neilsen, 1983, Vol. 30, Danish Medical Bulletin)

heim et al., 1976). The inhibitory concentrations of citrate on PFK in vitro have been shown to be within the physiological range (0.05 - 1.0 mM). Tissue differences in the sensitivity of PFK to inhibition by citrate related to the isoenzymes of PFK which has M, L and F subunits (Vora et al., 1980). The nature of PFK in human erythrocytes is in hybrids of subunits. M4-form of PFK found in skeletal and heart muscle was reported to be more sensitive to inhibition of citrate than others. Plasma citrate is released from skeletal muscle, bone and (or) skin. Bone has most often been stated to be a major source of citrate release to plasma. The skeletal muscles with its high capacity for oxidative substrate utilization is probably a source of citrate release across the working leg to the plasma pool (Nielsen et al., 1979). Glycolysis in skeletal muscle is thought to be controlled at the level of PFK. Changes in plasma citrate during exercise reflect variations in cytosolic citrate level of muscle and is the regulatory role on glucose oxidation in skeletal muscle. During continuous submaximal exercise, a decrease in muscular cytosolic citrate as in plasma citrate might be expected. This would activate PFK and oxidation of carbohydrate is known to be dominant (Nielsen, 1978). There is substantial evidence that increased oxidation of fatty acids and ketone bodies inhibits glucose oxidation in rat by inducing a citrate inhibition of glycolysis at the level of PFK. On the basis of animal experiments, a citrate inhibition of PFK has been purposed as an additional

mechanism behind insulin deficiency as a cause of decreased glucose utilization in diabetics. Circulating concentration of citrate can be used as an estimate of the metabolic abnormality, in particular of the increased lability of blood glucose in diabetics. The lower mean plasma citrate level was found after increased insulin doses in diabetes. In insulinated diabetes, long-term insulin administration depressed plasma citrate level for improving cellular glucose utilization (Thomassen, Nielsen and Sorensen, 1981). Finding that plasma citrate rose continuously during fasting and urinary citrate excretion decrease indicate an accumulation of plasma citrate to decrease glucose utilization of some tissue (skeletal muscle and kidney cortex). This adaptation is of vital importance for conserving available glucose for glucose requiring tissues (Nielsen and Sorensen, 1979).

OBJECTIVES OF RESEARCH

The proposes of the studies are

- (1) to define the action of chemicals commonly used in blood sampling and storage on intraerythrocytic fructose-1,6-diphosphate (FDP), an important cellular metabolic regulator, and
- (2) to follow changes of this metabolic regulator in the red blood cells of Diabetic and Thalassemia patients.

MATERIALS AND METHODS

Animals and Human Subjects

Adults male rats of Wistar strain, weighing 180-250 gm were used in the studies. They were obtained from the National Laboratory Animal Center of Mahidol University. Upon arrival at the Faculty of Science 5 to 7 rats of the same sex were housed together in stainless-steel cage with water and rat chow ad libitum. They were left to be acquainted to the new environment at room temperature for a few days before the experiment. Rats were put under anesthesia with pentobarbital (50mg/kg, i.p.) while blood sampling were taken from the femoral artery (approx. 6 to 8 ml) into a plastic syringe containing appropriate anticoagulant.

Blood samples of human volunteers were collected into a 15 ml-test tube, containing either acid-citrate-dextrose or heparin as anticoagulant at the blood bank, Ramathibodi Hospital. Those of diabetic and thalassemia patients were from O.P.D. wards, departments of medicine Ramathibodi Hospital and Thalassemia center, Siriraj hospital respectively. Both male and female subjects were used in the studies.

Copyright by Mahidol University

B. Chemicals

The reagents were purchased from the following sources and used without further purification. Sodium dihydrogenphosphate 2 hydrate and potassium carbonate from Riedel De Haen AG, Seelze-Hannover, Germany; disodium hydrogen phosphate anhydrous from BDH Chemicals Ltd, Poole, England; sodium chloride and perchloric acid (70% w/w, sp.gr. 1.67) from E.Merck, Darmstadt, Germany; sodium citrate dihydrate from J.T.Baker Chemical Co., Phillipsburg N.J., U.S.A.; citric acid monohydrate from Calbiochem, San Diego, California, U.S.A.; dextrose anhydrous from Mallinckrodt, St. Louis, Missouri, U.S.A.; heparin(5000 iu/ml) from Leo pharmaceutical Products, Denmark; Nembutal sodium from Abbott Laboratories North Chicago, U.S.A.; Anhydrous Tris-HCl, reduced nicotinamide adenine dinucleotide disodium salt (NADH- Na_2), glycerol-3-phosphate dehydrogenase/ triosephosphate isomerase (GDH 150 $\bar{\text{u}}$ /mg protein with TIM 1250 $\bar{\text{u}}$ /mg protein), aldolase (13 $\bar{\text{u}}$ /mg protein) from Sigma Chemical Co., Saint Louis, Mo., U.S.A.; and fructose-1,6-diphosphate trisodium salt (Esafosfina) from Biomedica Foscama, Italy.



C. Preparation of Reagents

C.1 ACD, CPD, SALINE, PBS and PBS-D

The Acid Citrate Dextrose (ACD) solution contains 8.0 gm of citric acid, 22.0 gm of Sodium citrate and 24.5 gm of glucose per liter in water. Citrate Phosphate Dextrose (CPD) a modified ACD solution, is slightly less acidic. It contains 3.27 gm of citric acid, 23.6 gm Sodium citrate, 25.5 gm of glucose and 2.22 gm of Sodium dihydrogenphosphate per liter in water (Williams, Beutler, Erslev and Lichtman, 1983). Normal Saline (0.9% NaCl) was obtained from local drugstore. Phosphate buffer-saline solution (PBS) was prepared by mixing 2 ml of 0.5 M Sodium dihydrogen phosphate, 8 ml of 0.5 M Disodium hydrogen phosphate with 8.81 gm sodium chloride in 1000 ml water and pH of the buffer was adjusted with proper phosphate salt accordingly. One gram of dextrose was added into a liter of PBS to make 0.1g% dextrose in PBS or PBS-D.

C.2 Standard FDP

Four standard FDP solutions containing 4.184 μg , 2.092 μg , 1.046 μg , 0.523 μg of FDP in 50 μl were prepared. They were

Copyright by Mahidol University

accurately prepared by diluting stock solution (dissolved 75 mg of FDP sodium in 10 ml of water) with water. 10 μ l of stock solution diluted to 1.5 μ l would be contained 4.184 μ g FDP/50 μ l and then further diluted by serial dilution (1:1) in 3 serial dilution steps.

C.3 Reagents for the assay of FDP

Enzymes GDH/TIM and Aldolase with specific activity of 150 units/mg protein and 13 units/mg protein respectively were diluted with water before used. The 10 μ l of diluted GDH/TIM solution containing 7 units GDH/mg Protien and 5.7 units TIM/mg protien was used in each assay tube. Aldolase was diluted to 2.3 units/mg protien (50 μ l of enzyme suspension diluted with 0.283 ml of water). 7.1 mg of NADH (M.W. 709.4) must be dissolved in water and bring up to 2 ml to obtain 5 mM of NADH solution. Tris HCL buffer (M.W.158, 0.1 M) was used as standard buffer and NaOH was used for pH adjustment in assay system.

Stability of Solutions

Keep all solutions in stoppered containers in a refrigerator at 4-10°C and the stock solution of FDP frozen. Under these conditions, the diluted enzyme solutions can be kept for

one week. NADH solution is stable for 2-3 weeks. Tris HCL buffer (0.1 M, pH 7.6) is stable for approximately 1 month at 4°C. Standard FDP solution were freshly prepared from stock solution which can be kept infinitely at 0°C.

D. Collections and Preparations of Blood Samples

D.1 Animals

Rats were anesthetized intraperitoneal (ip.) injection with Nembutal sodium (50 mg/kg). Blood samples were drawn from femoral artery with heparinized plastic syringes. About 5-6 ml of rat blood was centrifuged at 500g for 10 min in a refrigerated (4 °C) centrifuge. After removing the plasma and upper buffy coat, the red cells were washed 3 times with PBS (pH 7.4) and the packed RBC (pak-RBC) was accordingly obtained. Aliquots of 1 ml of pooled pak-RBC from 9-10 sacrificed rats were immediately deproteinized at time 0 hr, incubated for 1 hr and 24 hr in refrigerator with the inclusion of ACD pH 4.8; CPD pH 5.5; PBS pH 7.4, 4.8, 5.5, 6.4; PBS-D pH 7.4 and Normal Saline pH 6.4 in the ratios 1 ml of pak-RBC to 0.5 ml of the designated medium or only by pak-RBC alone.

One ml aliquots of pooled pak-RBC from 2 sacrificed rats was incubated at 1 hr with citrate buffer (citric acid and Sodium

citrate), varying pH from 8.5, 6.4, 5.5 to 4.8.

At indicated incubation time, samples were removed from the refrigerator and immediately deproteinized with 5 ml of 6% (w/w) perchloric acid then centrifuged at 3000 rpm for 10 min. Four ml of the supernatant, were neutralized with 2.5 ml of K_2CO_3 (0.5 M) and kept settled for 30 min. The neutralized supernate was saved for later analysis of FDP and kept in the refrigerator. The solution can be kept unaffected for at least one week (Michal and Beutler, 1974a).

D.2 Human

Human blood was collected from healthy volunteers with ACD (0.15 ml : one ml of whole blood) or with heparinized tube. About 10-15 ml of whole blood was centrifuged at 500g for 10 min in a refrigerated (4 °C) centrifuge to separate the red cells from plasma and other cells in the buffy coat. The collected RBC's were washed 3 times with cold PBS (pH 7.4) to obtain pure pak-RBC. Each 1 ml of the pak-RBC collected with ACD or heparin was deproteinized as eariler described in rat at time 0 hr or further incubated in refrigerator for 1 hr and 24 hr with PBS-D. At the indicated intervals, the samples were immediately removed and deproteinized.

Copyright by Mahidol University

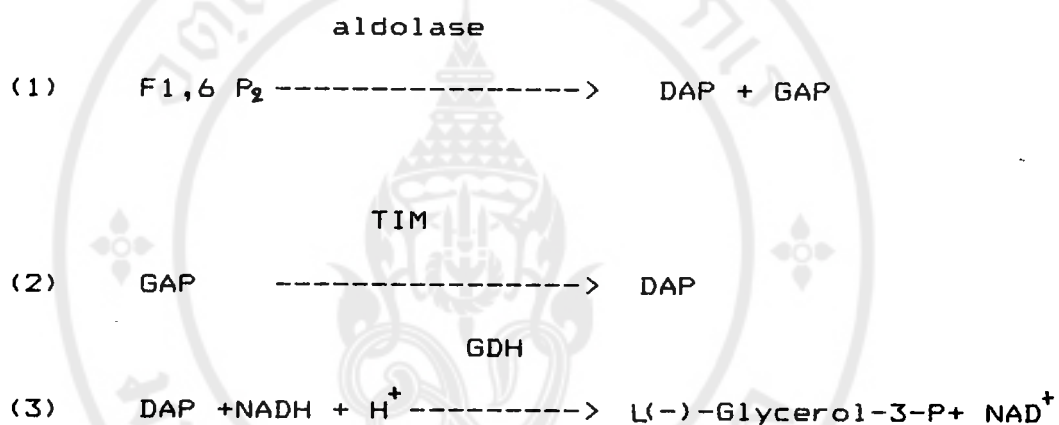
Human volunteers, diabetics and thalassemia blood were collected with heparin. About 10-15 ml of blood was centrifuged to separate the red cells as described earlier. One ml of the obtained pak-RBC from either normal or patients was deproteinized at 0 hr or further incubate with PBS-D in ratio 1 ml of pak-RBC : 0.5 ml of PBS-D for 1 hr and 24 hr. At those intervals of incubation the samples were immediately deproteinized with perchloric acid and supernate was obtaining following centrifugation.

In all of experiments blood samples were always kept ice cool before assay. All reagents which were used in this experiment must be stored in refrigerator and continuously controlled in ice cool bath while FDP extraction was being prepared for the incubated blood samples.

E. Determination of Fructose 1,6 diphosphate

The amount of FDP in supernate deproteinized pak-RBC was measured by enzymatic method, as readily documented by Michal and Beutler (1974,b).

E.1 Principle



Coupling of reaction



Aldolase (D-Fructose-1,6-bisphosphate, D-glyceraldehyde-3-phosphate lyase: E.C.4.1.2.13 cleaves fructose-1,6-diphosphate (F-1,6- P_2) into glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DAP).

Triosphosphate isomerase, TIM (D-Glyceraldehyde-3-phosphate keto-isomerase, E.C. 5.3.1.1) catalyses the equilibrium between GAP and DAP.

Glycerol-3-phosphate dehydrogenase, GDH (sn-Glycerol-3-phosphate : NAD 2-oxidoreductase, E.C.1.1.1.8) catalyses the reduction of dihydroxyacetone phosphate (DAP) with reduced nicotinamide-adenine dinucleotide (NADH)

The reaction sequence proceeds rapidly with an excess amounts of NADH and adequate enzyme concentrations. The decrease in extinction at 340, 334, or 365 nm associated with the oxidation of NADH is measured. 2 moles of NADH are oxidized per mole of F-1,6-P₂. The inclusion of the triosephosphate isomerase reaction (2) thus increases the sensitivity of the determination.

E.2 Assay protocol

All reagents were always in ice cool bath. The enzymatic reaction of analysis was started by incubating the mixture at room temperature in a quartz cuvette. Using 340 nm to be optimum wavelength. Cuvette tubes were allowed to stand in spectrophotometric chamber of a double beam spectrophotometer (UVIDEC-650; JASCO). Read the extinction value against air. A four point standard curve with added zero point (using water rep-

resent to standard FDP zero conc.) were done for many times to assure the reproducibility. The measuring method was basically followed the decreasing in absorption at 340 nm, resulted from the oxidation of NADH.

Neutralized supernates of deproteinization blood were determined for FDP level.

Assay system

Pipette 3 ml of Tris HCl buffer (pH 7.6) into a cuvette and draw off the anticipated volume to be replaced by other reagents. Add 10 μ l of NADH solution (5 mM), 10 μ l of GDH/TIM enzyme solution and 50 μ l of standard FDP into cuvette buffer tube. Mix the contents in the cuvette thoroughly well before measured. Immediately measure the Extinction₁ (E_1) at time 0 and set up the time. The reaction would be complete in 8-10 mins. At the end of the incubation period, measure the E_2 value before adding 10 μ l of aldolase and mix it again then measured the E_3 20 min later, after the reaction occurred completely measure E_4 .

Unknown samples were analyzed as the same procedure, but must be adjusted optimum volume which can be determined within the range of the standards but not more than 2 ml.

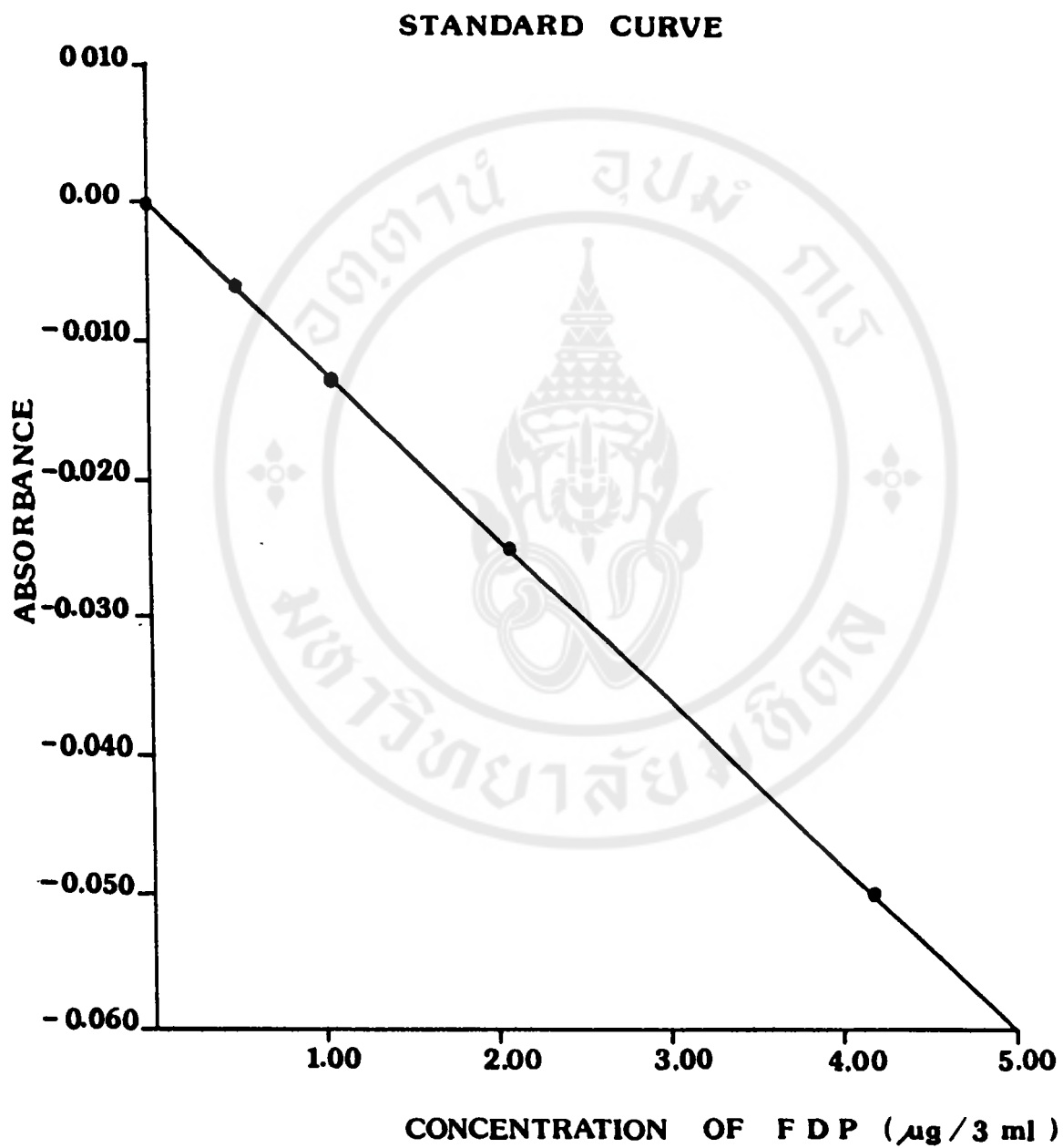


Fig 4 : Standard curve of Fructose 1,6 diphosphate

SCHEME

pipette into cuvettes:

Tris HCl buffer pH 7.6	3 - #	ml
NADH	10	μ l
GDH/TIM	10	μ l

standard FDP 50 μ l or unknown sample
mix, bring to room temperature,
and read initial extinction E_1
After the reaction has occurred (8-10 min)
read E_2 several times.

$$E_1 - E_2 = \Delta E \text{ DAP and GAP} *$$

Aldolase	10	μ l
----------	----	---------

mix and read E_3 .

After the reaction has occurred (20 min)
read E_4 several times.

$$E_3 - E_4 = \Delta E (F1,6-P_2)$$

Volume of NADH, GDH/TIM, Aldolase, and unknown sample or
standard added into cuvette

* Even if only fructose diphosphate was to be determined, this
sequence should be followed, i.e. dihydroxy acetone phosphate
(DAP) and glycerol-3-phosphate (GAP) present in the extract
must be reduced before the FDP determination can be started.

Copyright by Mahidol University

E.3 Calculation

The concentration of FDP in unknown samples were determined from $\Delta(E_3-E_4)$ interpolating to the standard curve. It was presented in the term of μg FDP per volume of unknown extracted sample which was added in procedure. Get the obtained determination calculated further by multiplying with dilution factor to be presented in μg FDP per 1 ml of pak-RBC.

Dilution factor equaled to the plus volume of HClO_4 (6% w/w, 5 ml) which was added for deproteinizing 1 ml of pak-RBC multiplying with the plus volume of K_2CO_3 (2.5 M, 0.5 ml) and 4 ml of supernate deproteinization and divided to the part of volume which was added in cuvette and 4 ml of supernate. As shown in this equation:

$$\text{FDP } (\mu\text{g/ml pak-RBC}) = \frac{\text{conc. of FDP from the curve} \times 4.5 \times 6}{\text{vol. of sample added in cuvette} \times 4}$$

Statistical Analysis

The mean ($\bar{X}$) and standard deviation (SD) for each set of data were calculated statistically. Student's t-test was used as method in testing the significant difference of the data. determined level of significance was set at the level of $P \leq 0.005$ or $P \leq 0.01$.

Copyright by Mahidol University

RESULTS

A. Effect of citrate and buffers on intraerythrocytic FDP

This experiment was designed to study the effect of citrate on intraerythrocytic FDP by incubating 1 ml of pooled rat pak-RBC (packed RBC) with citrate in vitro. NaCl, PBS with and without glucose pH 7.4, and PBS with varied pH's corresponding to the pH's of ACD (4.9), CPD (5.3) and NaCl (6.3) were served as paralleled controls. After incubated pak-RBC for 1 hr and 24 hr, the assigned tubes were deproteinated and supernated prepared for FDP assay.

Citrate in both ACD and CPD showed inhibitory effect on glycolytic pathway of RBC (Figure 5 and 6). FDP level was significantly lower in the RBC incubated with either ACD or CPD than those in PBS at both after 1 hr and 24 hr incubations. The level in PBS pH 7.4, 4.9, 5.6, 6.3 and NaCl were not significantly different among themselves (Table 3). All have the same level of FDP after 1 hr and 24 hr incubation, but PBS-D at time 24 hr has the ability to maintain the FDP level better than PBS (Fig.7). Levels of FDP in RBC incubated with ACD and CPD are around 170 $\mu\text{g/ml}$ pak-RBC at 1 hr which is 50% reduction from PBS 7.4, and

Copyright by Mahidol University

Table 3 : Concentrations of Fructose 1,6 diphosphate in RBC in the Presence of Different Incubating Buffers.

condition	FDP ($\mu\text{g/ml}$ pak-RBC)		
	Incubation time (hr)		
	0	1	24
pak-RBC	246.11 $\pm$ 56.52*	319.27 $\pm$ 37.59 (+ 29.73)	128.25 $\pm$ 39.44 b (- 47.89)
PBS(pH 7.4)		322.82 $\pm$ 7.06 (+ 31.17)	127.75 $\pm$ 16.63 b (- 48.09)
ACD(pH 4.8)		171.54 $\pm$ 14.42 a (- 30.30)	2.65 $\pm$ 0.46 a,b (- 98.92)
CPD(pH 5.5)		165.41 $\pm$ 18.29 a (- 32.79)	2.59 $\pm$ 0.24 a,b (- 98.95)
PBS-D(pH 7.4)		328.46 $\pm$ 19.63 (+ 33.46)	218.96 $\pm$ 13.41 a,b (- 11.03)
PBS(pH 4.8)		324.24 $\pm$ 12.51 (+ 31.74)	121.87 $\pm$ 19.07 b (- 50.48)
PBS(pH 5.5)		327.07 $\pm$ 16.63 (+ 32.90)	119.06 $\pm$ 17.87 b (- 51.62)
PBS(pH 6.4)		319.99 $\pm$ 16.85 (+ 30.02)	131.74 $\pm$ 18.64 b (- 46.47)
NaCl(pH 6.4)		321.40 $\pm$ 18.74 (+ 30.59)	135.31 $\pm$ 17.14 b (- 44.37)

Values are means $\pm$ S.D., n = 4

*is the level of FDP before incubation, n = 3

Values in the parentheses are per cents increased(+) or decreased(-) of RBC FDP from the level before incubation.

a = significantly different from the pak-RBC FDP in each column,
P $\leq$ 0.005

b = significantly different from FDP at 1 hr in each row,
P $\leq$ 0.005

Copyright by Mahidol University

Effect of ACD on the levels of FDP in Rat RBC

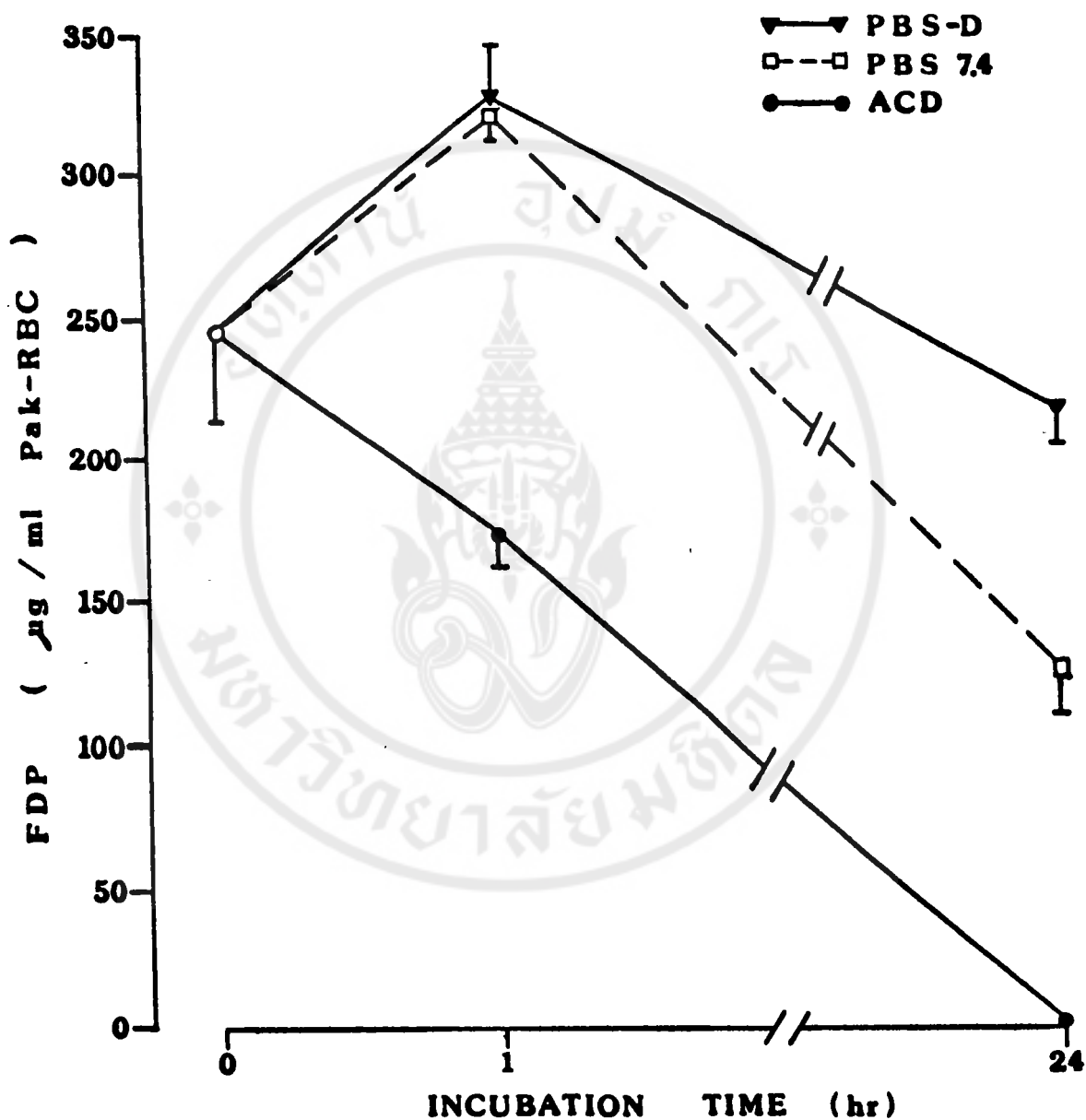


Fig 5 : Pooled of rat packed RBC, collected with heparin, in aliquots of 1 ml were incubated in vitro at 10°C at various intervals in the presence of either ACD, PBS-D or PBS pH 7.4.

Effect of CPD on the levels of FDP in Rat RBC

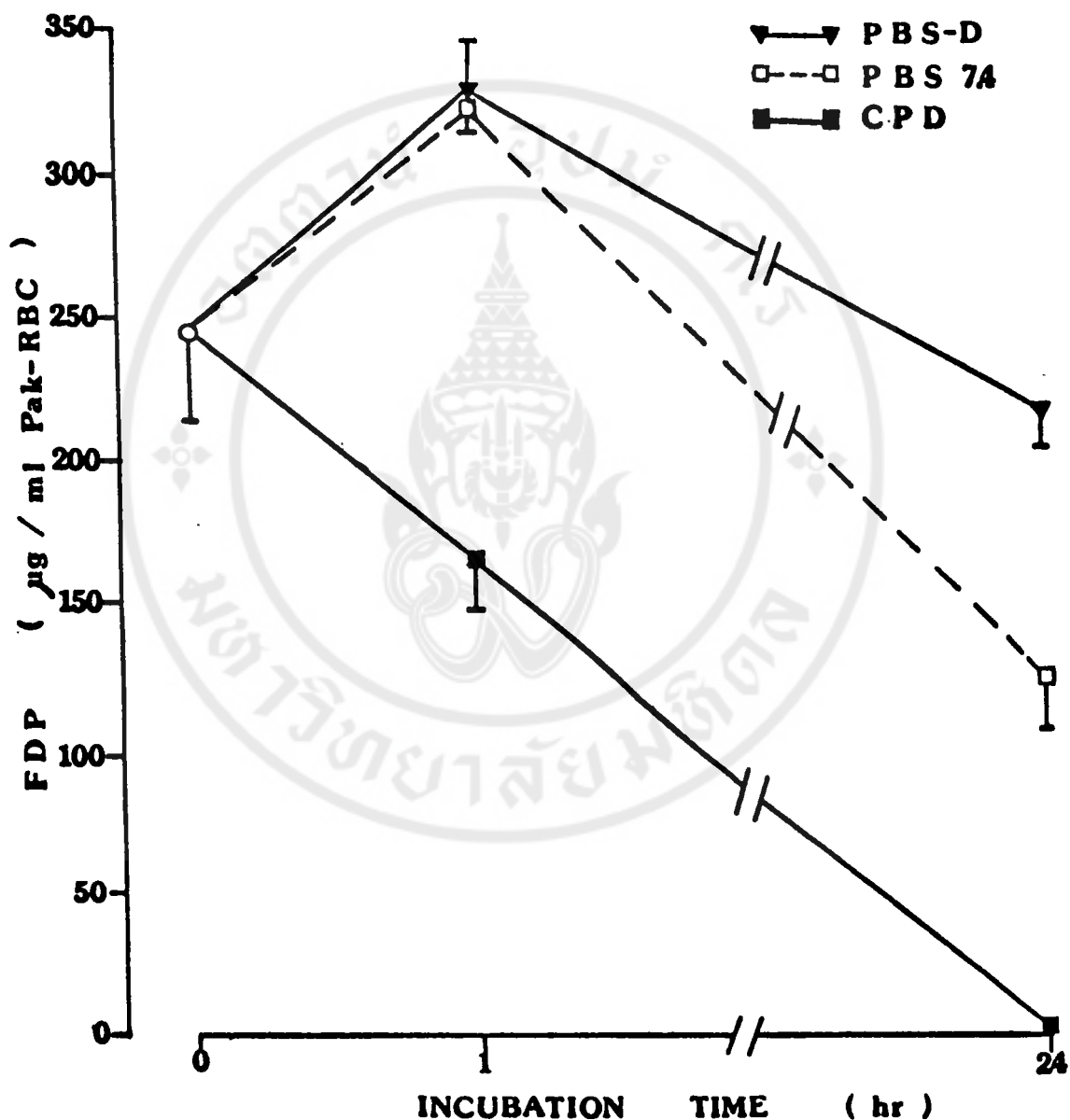


Fig 6 : Pooled of rat packed RBC, collected with heparin, in aliquots of 1 ml were incubated in vitro at 10°C at various intervals in the presence of either CPD, PBS-D or PBS pH 7.4.

Levels of FDP in Rat RBC incubated In vitro

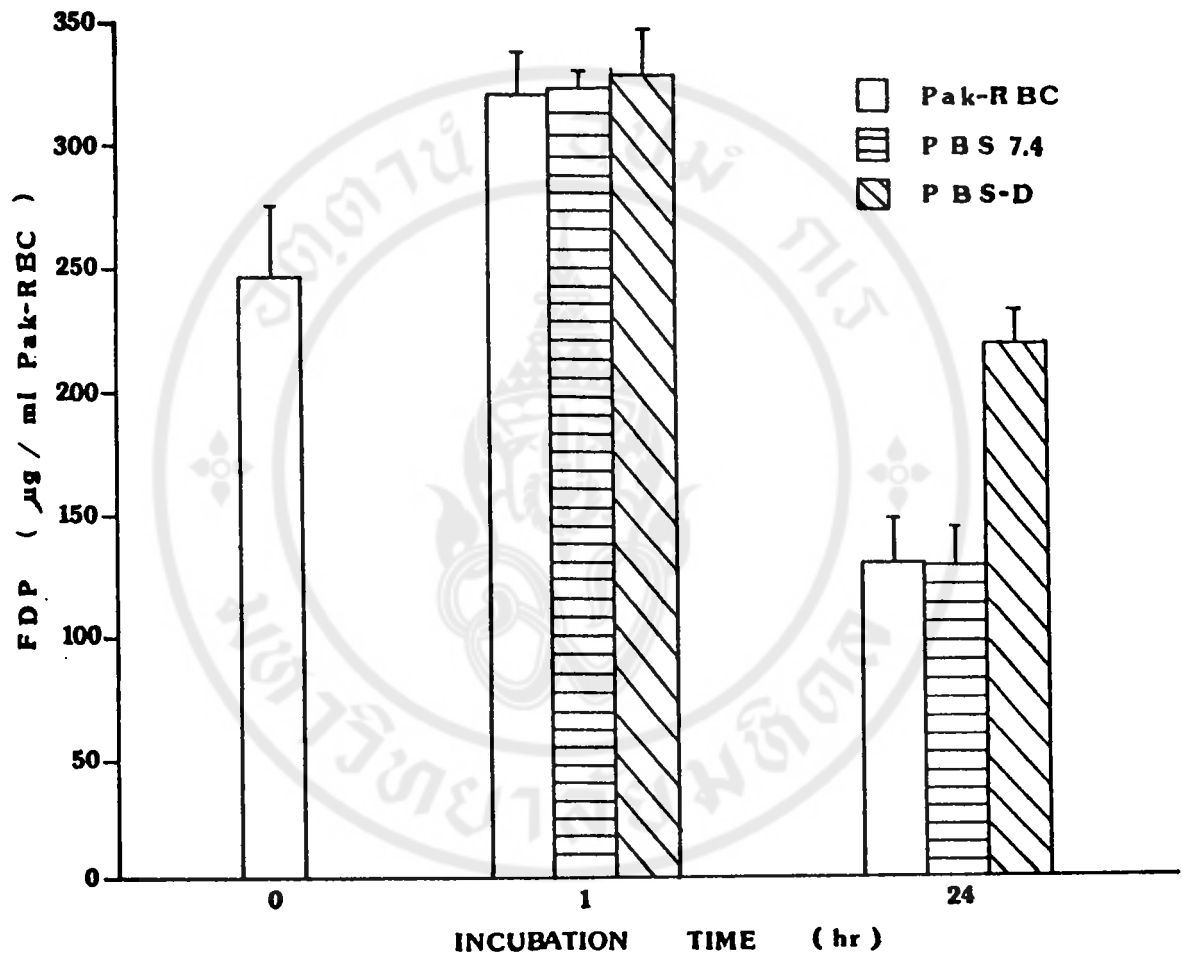


Fig 7 : Aliquots of 1 ml packed RBC, collected with heparin, were incubated with PBS-D and PBS pH 7.4, compared to FDP in 1 ml of packed RBC at various intervals.

* at time zero it is the basal level of FDP

a highly significant decrease to 2.5 $\mu\text{g/ml}$ pak-RBC at 24 hr. In PBS-D, FDP level at 1 hr incubation was about 328 $\mu\text{g/ml}$ pak-RBC (not significantly different) and 219 $\mu\text{g/ml}$ pak-RBC at 24 hr which was significantly higher than those in pH 7.4 PBS.

A.1 Effect of pH in citrate buffer

Citrate solutions in various pH (pH 8.5, 6.4, 5.5 and 4.8) were prepared. Each 1 ml of pooled rat blood was incubated in different citrate solutions using the corresponding pH of PBS as controls. Decreasing the pH of citrate buffer increased the inhibitory effect of citrate on amounts of FDP in the incubated pak-RBC, as shown in table 4. The effect was not seen with PBS at the matched control pH's, Fig. 8. Per cent reduction of FDP in different citrate solution is showned in Fig. 9.

A.2 Comparative effects of ACD and Heparin

Both ACD or heparin have been clinically used as anticoagulant. In this study, the effect of citrate in ACD on FDP level in human blood during collecting blood followed and compared with heparin. Whole blood collected with ACD or heparin were separated to obtain pak-RBC as described, the levels of FDP in 1 ml pak-RBC were determined at zero time (within 2 hr of the blood sample preparation/without incubation with any assigned

Table 4 : FDP levels in Packed RBC after Incubate with Various pH Values of Citrate and PBS, and ACD for 1 hr.

pH value	FDP ($\mu\text{g/ml}$ Pak-RBC)		
	Incubated for 1 hr.		
	Citrate	PBS	ACD
8.5	273.38 $\pm$ 32.46		
6.4	250.80 $\pm$ 38.43 a	319.99 $\pm$ 16.85	
5.5	155.77 $\pm$ 40.81 a	327.07 $\pm$ 16.63 b	
4.8	133.18 $\pm$ 26.01 a	324.24 $\pm$ 12.51 b	141.06 $\pm$ 44.66
7.4		328.46 $\pm$ 19.63	

Values are means $\pm$ S.D., n = 4

a = significantly different from FDP in citrate pH 8.5, $P \leq 0.01$

b = significantly different in each row, $P \leq 0.01$

Table 5 : Effect of pH in Citrate Buffer on the Levels of FDP

Incubated with	Incubated for 1 hr.	
	FDP ($\mu\text{g/ml}$ pak-RBC)	per cents decreased
Citrate pH 8.5	273.38 $\pm$ 32.46	0.00
Citrate pH 6.4	250.80 $\pm$ 38.43	8.26
Citrate pH 5.5	155.77 $\pm$ 40.81	43.02
Citrate pH 4.8	133.18 $\pm$ 26.01	51.28

Effect of Citrate and pH on FDP Level in Rat RBC

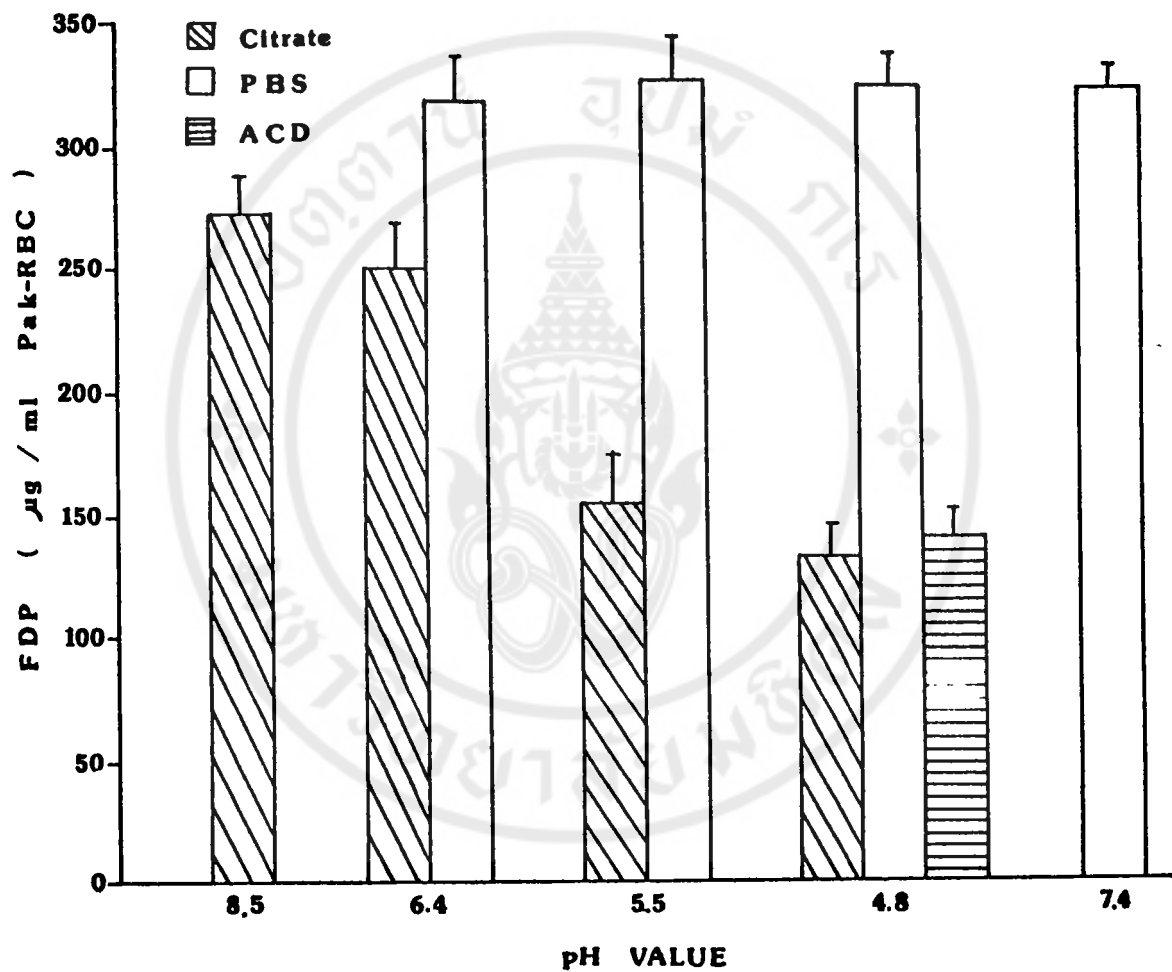


Fig 8 : Rat packed RBC, collected with heparin, in aliquots of 1 ml were incubated in vitro at 10°C for 1 hr in the presence of citrate buffer or phosphate buffer saline at various pH.

% Decrease of FDP in various pH of Citrate

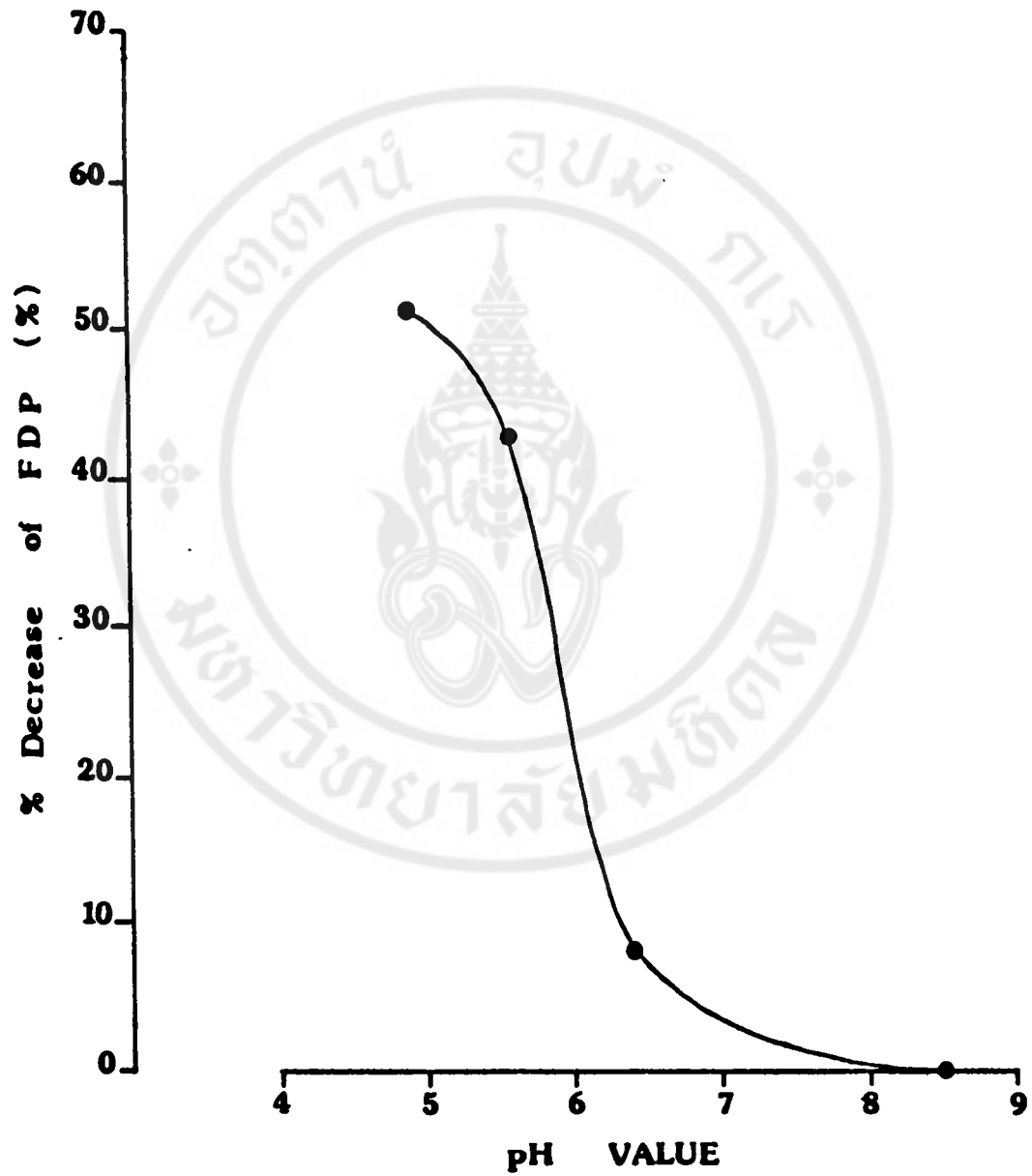


Fig 9 : Effect of pH in citrate buffer on per cent reduction in rat packed RBC

buffer). Some parts of the prepared pak-RBC were further incubated for 1 hr and 24 hr with PBS-D. The similar aliquots of heparinized blood were also processed and incubated accordingly. The results are shown in Fig. 10. At the zero time, level of FDP collected with ACD is significantly lower than that of heparin. PBS-D increased the FDP level in 24 hr of incubation, very highly significant elevated in blood collected with heparin. The potentiating effect of PBS-D on FDP production was less in blood collected with ACD than in blood collected with heparin. It is about 18 $\mu\text{g/ml}$ pak-RBC significantly less than FDP level in heparinized blood. It seems likely that inhibitory effect of ACD on FDP production in red blood cell is almost complete. FDP level of human RBC incubated with PBS 7.4 (without dextrose) was not potentiated at 24 hr unlikely that in condition of PBS-D incubation. Pattern of changes in FDP level in human RBC incubated with PBS in the presence and absence of dextrose were shown in Fig. 11.

Effect of Anticoagulant (Heparin or ACD) on Human RBC

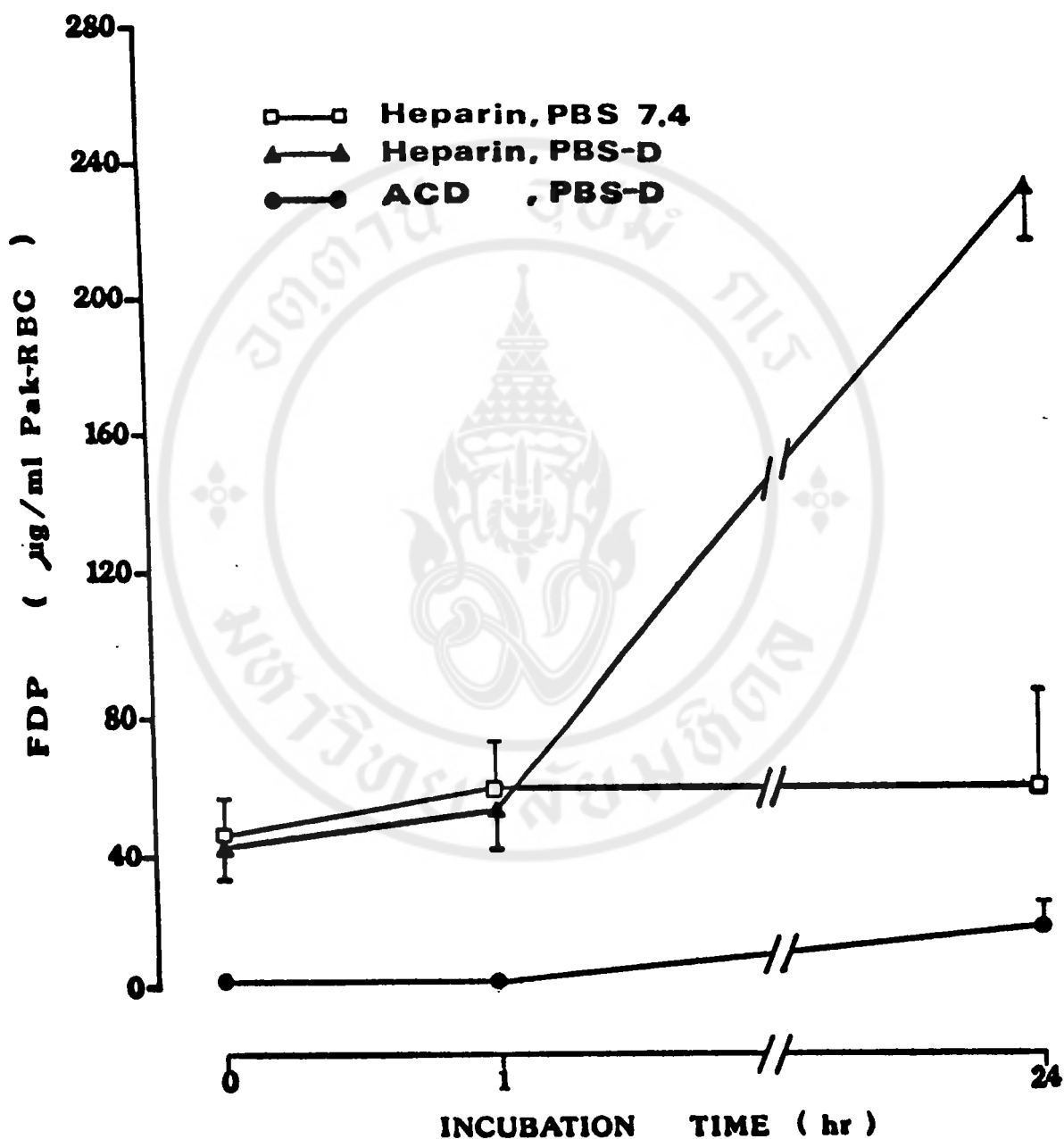


Fig 10 : Human RBC, collected with ACD or heparin as anticoagulant, were washed 3 times with PBS pH 7.4 at 4°C and incubated with PBS-D pH 7.4 in vitro at 10°C for 1 hr and 24 hr. (□—□ incubated with PBS pH 7.4)

Effect of PBS-D on FDP levels in Human RBC

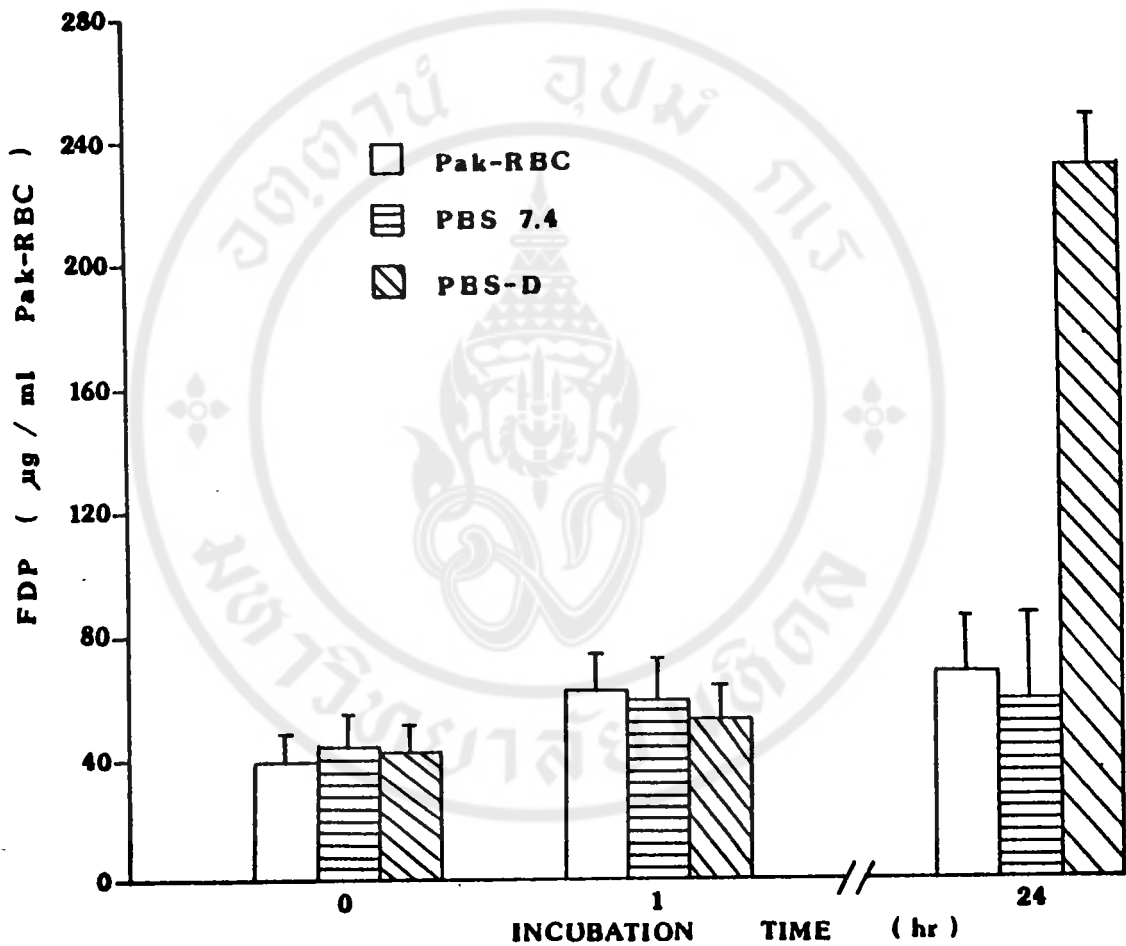


Fig 11 : Human packed RBC, collected with heparin, were washed 3 times with PBS pH 7.4 and then incubated in vitro at 10°C for 1 hr and 24 hr in the presence of either PBS-D pH 7.4 or PBS pH 7.4.

B. Levels of Intraerythrocytic FDP in Health and Disease

In this study, fresh blood from healthy volunteers, thalassemia and diabetes patients were prepared and the basal FDP levels were determined (0 hr). Some parts of pak-RBC were incubated with PBS-D 7.4 for 1 hr and 24 hr. All of the results are shown in table 6, 7, 8, 9 and Fig. 12.

B.1 Fresh blood from volunteers

In Table 6, the mean basal FDP level of 11 normal volunteers was about 36.00 $\mu\text{g/ml}$ pak-RBC. After incubated 1 ml of pak-RBC with PBS-D, FDP level increased to 42.16 $\mu\text{g/ml}$ pak-RBC at 1 hr and 225.14 $\mu\text{g/ml}$ pak-RBC at 24 hr. The percents changes of FDP production from the basal level were 17.11% and 525.39% at 1 hr and 24 hr, respectively. PBS-D significantly potentiate the FDP level.

B.2 Bloods of Diabetic patients

Basal level of FDP in RBC of DM patients was significant different from normal (Table 7). Diabetic red cells has a higher basal level of FDP than normal. After 1 hr incubation, the level of FDP was to the level found in normal blood and followed by a steady fall at the similar rate as in healthy volunteers blood in

the next 24 hr of incubation (Fig. 12). FDP production of diabetic red cells incubated with PBS-D at 24 hr was highly potentiated as similarity of the effect of PBS-D in normal blood.

B.3 Thalassemia

B.3.1 Nonsplenectomized

In nonsplenectomized type of thalassemic patients, the results are shown in table 8. The basal FDP level is about 147.37 $\mu\text{g/ml}$ pak-RBC, and FDP production of red cell incubated with PBS-D for 1 hr is about 164.04 $\mu\text{g/ml}$ pak-RBC while normal red cell is significantly lower with respectively comparable. And when incubate pak-RBC of βE -nonsplenectomized with PBS-D for 24 hr, the potentiate effect of PBS-D is less than normal blood. Percentage increase of FDP production at 24 hr is 70.08% significantly less than normal. The line graphs in Fig.12 indicates the different pattern in the changes of FDP level between βE thalassemia nonsplenectomized and normal volunteers.

B.3.2 Splenectomized

In experiment of βE thalassemia splenectomized type, the results indicate the interesting point and show in table 9. The red cells of βE thalassemia splenectomized have a higher basal

level of FDP than normal blood similar in β E thalassemia nonsplenectomized. But then incubated red cells of β E splenectomized for 24 hr, the FDP production was significantly decreased from 1 hr of incubation. This point is contrast different from β E nonsplenectomized. Fig. 12 show the different pattern of line graph between normal and β E thalassemia both splenectomized and nonsplenectomized. All these results indicate some defect was occurred in β E thalassemia, particular in splenectomized.

Table 6 : Levels of FDP in RBC of Normal Volunteers after Incubated in vitro at 10°C with PBS-D for 1 hr. and 24 hr.

FDP (ug/ml pak-RBC)				
ID. No	Incubation time (hr)			per cent changes *
	0	1	24	
V-054	27.58	33.83	225.65	+718.17
V-056	42.22	50.69	253.94	+501.47
V-057	56.36	56.36	225.65	+300.37
V-058	18.02	27.05	208.71	+1058.21
V-059	33.83	32.71	169.16	+400.03
V-060	27.05	29.32	197.44	+629.91
V-061	19.16	22.55	214.38	+1018.89
V-062	59.16	67.64	259.44	+338.54
V-063	36.59	45.06	236.99	+547.69
V-064	36.59	45.06	259.54	+609.32
V-065	39.42	53.53	225.65	+472.43
$\bar{X}$	36.00	42.16 a	225.14 a	+599.55
S.D.	+13.28	+14.16	+27.60	+250.44

* are per cents increased (+) or decreased (-) of RBC FDP at 24 hr. from the level before incubation (0 hr.)

a = significantly different from the mean of level before incubation, $P \leq 0.005$

Copyright by Mahidol University

Table 7 : FDP levels of Diabetic Patients at Various Times of Incubation Period in the Presence of PBS-D.

FDP(ug/ml pak-RBC)				
ID. No	Incubation time (hr)			per cent changes *

	0	1	24	
DM-001	73.29	42.22 (-42.39)*	191.77	+161.66
DM-002	78.94	45.06 (-42.92)*	239.69	+203.64
DM-003	76.11	-	220.05	+189.12
DM-004	67.64	42.22 (-37.58)*	210.51	+211.22
$\bar{X}$	74.00	43.17	215.51	+191.41
S.D.	+ 4.82	+ 1.64	+ 19.95	+ 21.85

* are per cents increased (+) or decreased (-) of RBC FDP at 24 hr. from the level before incubation (0 hr.)

a = significantly different from the mean of level before incubation, $P \leq 0.005$

Table 8 : Levels of FDP in RBC of Thalassemic Nonsplenectomized patients after Incubated in vitro at 10°C with PBS-D for 1 hr. and 24 hr.

FDP (ug/ml pak-RBC)				
ID. No	Incubation time (hr)			per cent changes *
	0	1	24	
T-015	76.11	107.19	259.34	+240.74
T-016	191.77	214.11	180.50	- 5.88
T-017	149.41	146.61	219.92	+ 47.19
T-018	186.10	197.44	293.22	+ 57.56
T-020	121.16	98.58	219.72	+ 81.35
T-021	121.16	135.27	242.50	+100.15
T-023	211.55	214.38	304.43	+ 43.90
T-025	112.73	138.14	304.43	+170.05
T-027	124.00	146.61	169.16	+ 36.42
T-029	157.88	186.10	259.54	+ 64.39
T-003	169.16	220.05	304.43	+ 79.97
$\bar{X}$	147.37	164.04 a	250.65 a	+ 83.26
S.D.	± 40.47	± 44.05	± 49.17	± 68.29

* are per cents increased (+) or decreased (-) of RBC FDP at 24 hr. from the level before incubation (0 hr.)

a = significantly different from the mean of level before incubation, $P \leq 0.01$

Table 9 : FDP levels of Thalassemic Splenectomized Patients at Various Times of Incubated Period in the Presence of PBS-D.

FDP(ug/ml pak-RBC)				
ID. No	Incubation time (hr)			per cent changes *

	0	1	24	
T-014	78.94	53.53	7.89	-90.01
T-002	98.72	84.49	12.38	-87.46
T-019	101.39	90.11	50.69	-50.00
T-022	90.11	63.33	21.41	-76.24
T-024	90.11	81.64	22.55	-74.98
T-028	132.47	138.11	129.67	- 2.11
T-030	90.11	132.47	7.32	-91.88
T-031	112.73	129.67	95.78	-15.03
T-033	107.06	107.06	23.67	-77.89
T-034	84.44	81.64	70.34	-16.70
T-035	103.30	81.64	132.47	+28.24
$\bar{X}$	99.03	94.88	52.20 a	-50.37
S.D.	±15.00	±28.29	±47.83	±41.94

* are per cents increased (+) or decreased (-) of RBC FDP at 24 hr. from the level before incubation (0 hr.)

a = significantly different from the mean of level before incubation, $P \leq 0.005$

Levels of FDP in Normal and Patients Human RBC

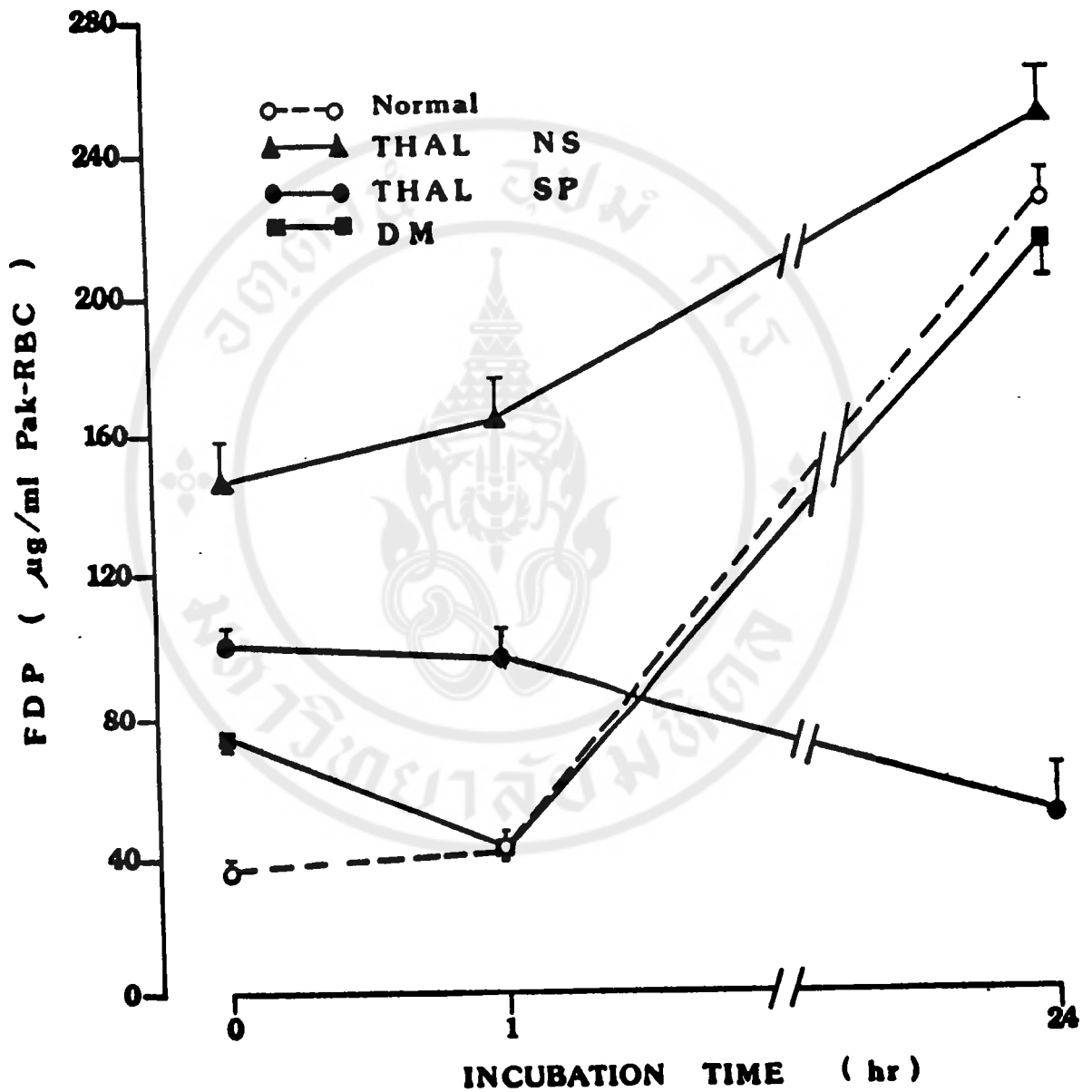


Fig 12 : Normal and patient packed RBC, collected with heparin, in aliquots of 1 ml were incubated in vitro at 10°C at various time in the presence of PBS-D.

DISCUSSION

Citrate and heparin have been used as anticoagulant in clinical blood sampling. Citrate is also used in blood storage. ACD and CPD are the two major constituents in preservative solutions and were often used in the past for keeping the whole blood or red cells. It contains glucose in citrate buffer at a pH of either 4.8 or 5.6. Blood collected in a heparin anticoagulant solution has relatively poor erythrocyte preservation since it lacks added glucose and tends to clot as the anticoagulant effect of heparin is neutralized during storage. Blood must be used within 48 hr of heparin collection (Masouredis, 1983). Because of these limitations, recalcified ACD or CPD blood has been adopted as a substitute for blood collected in heparin. However ACD and CPD are yet to be ideal. When whole blood or packed red cells are stored in either ACD or CPD, the concentration of red cell ATP falls gradually during storage. When the ATP level declines to 0.4 mM or less, the capacity of red cells to phosphorylate glucose is impaired and their viability is lost. Hemoglobin oxygen affinity and 2,3 DPG levels change rapidly in ACD blood. Some 40 percent of the 2,3 DPG is lost in the first week of storage, resulting in a significant increase in oxygen affinity. After 2 weeks of storage, virtually all of the 2,3 DPG disappeared from blood stored in ACD solution (Masouredis, 1983). Erythrocytic loss of deformability correlates to certain extent

with the loss of ATP (La Celle and Weed, 1971).

This major problem of ACD and CPD in blood storage may be due to the effect of citrate on the glycolytic pathway. These are some evidences showing that citrate is acting as allosteric modifier to the PFK. Citrate has been found to inhibit PFK in a variety of tissues, e.g., skeletal muscle, heart muscle and brain tissue (Nielsen, 1983). Citrate can potentiate the inhibitory effect of ATP, decrease the binding of the activators AMP, cAMP, ADP, F6P to crystalline PFK (Tornheim, et al., 1976; Colombo, et al., 1975). Citrate greatly reduced the maximum activation by FDP (Tornheim, 1985). The effect of FDP in promoting the in vitro phosphorylation of the enzyme is reduced in the presence of low concentrations of citrate (Foe, Laf Shaw, and Kemp, 1983). Addition of citrate in the physiological concentration (0.05-1.0 M) can markedly altered the V_{max} for FDP (Tornheim, 1985).

We studied the effects of citrate on FDP production in red blood cell. Incubation of ACD and CPD in vitro of both rat and human blood demonstrated that rate of FDP production in red blood cells was clearly suppressed. The inhibitory effect of citrate on FDP production in stored blood supported the idea that citrate suppressed the PFK activity in red blood cell. The effect could not be due to pH as incubation of RBC in PBS at corresponding pH's served as controls. Acidic pH in PBS did not ef-

fect on lowering the FDP level. The results in our experiment support the contention that citrate suppressed the enzyme PFK in RBC. The suppressive effect of citrate on intraerythrocytic FDP was pH dependent. Acidic citrate was more effective in lowering the level of FDP than the less acidic citrate. Since pH did not directly affect FDP production, it seems likely that acidic pH of citrate buffer elevated the unionized form of citrate manipulated by Henderson-Hasselbalch equation. The unionized form of citrate could be more permeable and pass through membrane of RBC and get into the cells at the more acidic medium.

Our results demonstrated that the presence of dextrose in PBS 7.4 elevated the FDP production in comparative with PBS no dextrose after incubated for 24 hr. Dextrose is the first substrate in the pathway of glycolysis. It directly produces the erythrocytic FDP. Dextrose is useful as an important component in preservative agents for blood storage.

The abnormal production of intraerythrocytic FDP in pathological state was studied in our experiments. In diabetic patients, FDP level was variably changed from normal at 0 hr of incubation. The higher level of intraerythrocytic FDP in diabetic patients at 0 hr could be due to the prior exposure of red cells to relatively high blood glucose in diabetic patients, so that the intraerythrocytic levels of FDP were significantly

elevated before the in vitro incubation. We found that the FDP production of diabetic blood after incubated in vitro with the assigned medium for both 1 and 24 hr were followed the pattern of FDP levels in normal blood. This indicates that red blood cells of diabetic patients does not show any defect in FDP production and consequently, it is not found abnormality in glycolytic regulation.

The abnormality of FDP production was noticed in thalassemia patients, particularly in splenectomized thalassemia. Levels of intraerythrocytic FDP in thalassemia were similarly elevated at pre and post 1 hr of incubation compared with normal, while the levels of intraerythrocytic FDP after 24 hr of incubation of the splenectomized and nonsplenectomized were in contrast difference. The FDP level of the splenectomized thalassemia at 24 hr of incubation dropped down from pre- and post-1 hr incubation. But the FDP production of nonsplenectomized thalassemia continuously elevated after 24 hr incubation. The patterns of FDP production between splenectomized, nonsplenectomized and normal were obviously different.

This result suggests that some glycolytic regulatory defect was occurred in the thalassemia red cells; especially in splenectomized thalassemia. The elevated basal FDP production in thalassemia red cell of both nonsplenectomized and splenectomized

patients may be resulted from the hyperglycemia in these Thal- patients. It was recognized some years ago that diabetes is a complication of homozygous β thalassemia (Weatherall and Clegg, 1981). Bannerman, et al. (1967) described a 41-year-old male with an intermediate form of β thalassemia who, in addition to diabetes mellitus, had hypopituitarism. Probably the commonest endocrine abnormality is pancreatic insufficiency with frank diabetes mellitus or at least biochemical evidence of abnormal glucose metabolism (Weatherall and Clegg, 1981). There is progressive damage to the islets cells as thalassemic children grow older and that diabetes is a relatively common complication of the disease. It is likely that iron deposition in the pancreas causes failure of insulin secretion (Weatherall and Clegg, 1981).

This defect in the FDP production in thalassemic red cell is interesting. Further search of the distinction in such defect and the cause of defect remain to be seen.

REFERENCES

- Anderson, B.B., Perry, G.M. and Clements, J.E.: Red cell enzyme activities in Thalassaemia. Br. J. Haematol. 57: 711-718, 1984.
- Araki, K., and Rifkind, J.M.: Age dependent changes in osmotic hemolysis of human erythrocyte. J. Gerontol. 35: 499, 1980.
- Bannerman, R.M., Keusch, G., Kreimer-Birnbaum, M., Vance, V.K. and Vaughan, S.: Thalassemia intermedia, with iron overload, cardiac failure, diabetes mellitus, hypopituitarism and porphyrinuria. Am. J. Med. 42: 476, 1967.
- Barnhart, M.I. and Henry R.L.: The Erythrocyte. Sick cell, PP. 15-28, 1984.
- Bellingham, A.J., and Grimes, A.J.: Annotation Red cell 2,3-di phosphoglycerate. Br. J. Haematol. 25: 555-562, 1973.
- Besis, M., Lessin, L.S., Beutler, E.: Morphology of the erythron. Hematology, ed. by W.J. Williams, pp. 271, McGraw-Hill, Inc, 1983 a.
- Beutler, E.: Energy metabolism and maintenance of erythrocytes. Hematology, ed. by W.J. Williams, pp. 332-334, McGraw- Hill, Inc, 1983.
- Beutler, E.: Red cell metabolism. A manual of Biochemical methods , ed. by E. Beutler, pp. 3-6, Grune & Stratton, Inc, 1984.
- Birchmeier, W. and Singer, S.J.: On the Mechanism of ATP-induced shape changes in human erythrocyte membrane. The role of ATP

J. Cell Biol. 73: 647-659, 1977.

Bonaventura, C., Bonaventura, J., Amiconi, G., Tentori, L., Brunori, M. and Antonini, E.: Hemoglobin Abruzzo [B143 (H21) His Arg.] Consequences of altering the 2,3- diphosphoglycerate binding site. J. Biol. Chem. 250: 6273- 6277, 1975.

Brewer, C.J., and Powell, R.D.: Hexokinase activity as a function of age of the human erythrocyte. Nature 199: 704, 1963.

Brown, P.R. and Parks, R.E.: Use of high pressure liquid chromatography to investigate the 'in vitro' reactions of human erythrocytes with guanosine. Analyt. Chem. 45:948, 1973.

Bunn, H.F., Haney, D.N., Kamin, S., Gabbay, K.H. and Gallop, P.M.: The biosynthesis of Human Hemoglobin A1c slow glycosylation of Hemoglobin in vivo. J. Clin. Invest. 57: 1652-1659, 1976.

Card, R.T., and Brain, M.C. : The Anemia of childhood: Evidence for a physiologic response to hyperphosphatemia N. Engl. J. Med. 288: 388-392, 1973.

Card, R.T., Mohandas, N., Perkins, H.A., and Shoet, S.B.: Deformability of stored red blood cells relationship to degree of packing. Transfusion 22: 96-101, 1982.

Chapman, R.G., and Schaumburg, L.: Glycolysis and glycolytic enzyme activity of aging red cells in man. Br. J. Haematol. 13: 665, 1967.

Colombo, G., Tate, P.W., Girotti, A.W. and Kemp, R.G.: Interaction of inhibitors with muscle phosphofructokinase J. Biol. Chem.

250: 9404-9412, 1975.

- Costrini, R., Candiani, A., Cifra, E., Battocchio, G., Panico, S., Simonetti, N. and Gasparatto, A.: Protective action of Fructose 1,6 diphosphate sodium salt towards erythrocyte fragility in patients under going extracorporeal circulation. *IRCS Med. Sci.* 8: 257-258, 1980.
- Dawson, R.B.: Blood Storage XXIII: 2,3-DPG Maintenance for six weeks in a CPD-Adenine Inosine Perservative with and without Methyline Blue. *Transfusion* 17: 238-240, 1977.
- Detralgia, M., Cook, F.B., Stasiw, D.M., and Cerny, L.C.: Erythrocyte fragility in aging. *Biochim. Biophys. Acta.* 345: 213, 1974.
- de Verdier, C.H., Strauss, D., Ericson, A., Akerblom, O. and Hogman, C.F.: Purine metabolism of erythrocytes preserved in adenine, adenine-inosine and adenine-guanosine supplemented media. *Transfusion* 21: 397, 1981.
- Dionigi, R., Bonera, A., Fossati, G.S., Dominioni, L. and Bondoli, A.: Effect of fructose-1,6-diphosphate infusion during total parenteral nutrition on exogenous insulin requirements, *IRCS Med. Sci.* 6: 530, 1978.
- Ericson, A., Niklasson, F. and de Verdier, C.: Metabolism of Guanosine in Human Erythrocytes. *Vox. Sang.* 48: 72-83, 1985.
- Erslev, A.J.: Anemia of chronic renal failure. *Hematology*, ed. by W.J. Williams, pp. 417, McGraw-Hill, Inc, 1983.

- Erslev, A.J.: Classification of erythrocyte disorders. Hematology ed. by W.J. Williams, pp. 406, McGraw-Hill, Inc, 1983.
- Foe, L.G, Lafshaw, S.P. and Kemp, R.G.: Binding of Hexose Bisphosphates to muscle phosphofructokinase. Biochemistry, 22: 4601-4606, 1983.
- Giordano, C. and Santo, N.G.: Metabolic aspects of fructose diphosphate in total parenteral nutrition. IRCS Med. Sci. 11: 173-174, 1983.
- Gonzalez, A.M., Sochor, M., Hotherhall, J.S. and Mclean, P.: Effect of experimental Diabetes on the activity of hexokinase in rat lens: An example of glucose overutilization in diabetes. Biochem. Biophys. Res. Commun. 84: 858-864, 1978.
- Grimes, A.J.: Normal and Abnormal pyruvate kinase. Human Red Cell metabolism, ed. by A.J. Grimes, pp. 131, 1980.
- Grimes, A.J.: Oxygen transport and glycolysis: blood storage. Human Red Cell Metabolism, ed. by A.J. Grimes, pp. 145, London, 1980.
- Grimes, A.J.: Regulation of glycolysis. Human Red Cell Metabolism ed. by A.J. Grimes, pp. 95, London, 1980.
- Gunn, R.B., Silvers, D.N. and Rosse, W.F.: Potassium permeability in B-Thalassaemia Minor Red blood Cells. J. Clin. Invest. 51: 1043-1050, 1972.
- Henderson, J.F., Fraser J. H., McCoy, E.E.: Methods for the study of purine metabolism in human cells in vitro. Clin. Biochem. 7: 339, 1974.

- Henderson, J.F., Zombar, G. and Burridge, P.W.: Guanosine triphosphate catabolism in human and rabbit erythrocytes. Role of reductive deamination of guanylate to inosinate. *Can. J. Biochem.* 56: 474, 1978.
- Hill, M.A. and Court, J.M.: Erythrocyte membrane fluidity in type 1 Diabetes Mellitus. *Pathology* 15: 449-451, 1983.
- Iapichino, G., Pesenti, A., Redrizzani, D., Solca, M. and Vesconi, S.: 2,3 diphosphoglycerate blood levels after infusion of fructose 1,6 diphosphate (FDP) in polytransfused patients *IRCS. Med. Sci.* 6: 157, 1978.
- Iapichino, G., Scherini, A., Maritano, M., Solca, M., Franzosi, M.G., Raddrizzani, D., Recchia, M. and Farima, M.L.: 2,3-diphosphoglycerate (DPG) blood levels in polytransfused patients: effects of inorganic and organic phosphate infusion. *IRCS Med. Sci.* 8: 231-232, 1980.
- Jande, J.H. and Cooper, R.A.: Hereditary spherocytosis. *Hematology*, ed. by W.J. Williams, pp. 547, McGraw-Hill, Inc, 1983.
- Kahn, A., Marie, J., Garreau, H. and Sprengers, E.D.: The genetic System of the L-type pyruvate kinase forms in man. Subunit structure, interrelation and kinetic characteristics of the pyruvate kinase enzymes from erythrocytes and liver. *Biochim Biophys. Acta.* 523: 59, 1978.
- Kanter, Y., Bessman, S.P. and Bessman, A.N.: Red cell 2,3 diphosphoglycerate levels Among Diabetic patients with and without vascular complications. *Diabetes*, 24: 724-729, 1975.

Copyright by Mahidol University

- Kirtley, M.E., McKay, M.: Fructose-1,6-bisphosphate, a regulator of metabolism. *Mol. Cell. Biochem.* 18: 141-149, 1977.
- Knox-Macaulay, H.H.M. and Weatherall, D.J.: Studies of Red cell Membrane function in heterozygous β thal and other hypochromic anaemias. *Br. J. Haematol.* 28: 277, 1974.
- Kong, C.M and Parks, R.E.: Incorporation of the purine moieties of guanosine and inosine analogs into nucleotide pools of human erythrocytes. *Biochim. Biophys. Acta.* 327: 282, 1973.
- Lacelle, P.L. and Weed R.I.: The contribution of normal and pathologic erythrocytes to blood rheology. *Prog. Hematol.* 7: 1, 1971.
- Lazzarino, G., Cattani, L., Costrini, R., Mulieri, L., Candiani, A., and Galzigna, L.: Increase of Intraerythrocytic Fructose-1,6-Diphosphate after Incubation of Whole Human Blood with Fructose-1,6-Diphosphate. *Clin. Chem.* 17: 42-45, 1984.
- Lazzarino, G., Costrini, R., Cattani, L. and Diana, M.: Plasma and erythrocyte concentrations of fructose 1,6 diphosphate after intravenous administration in humans. *IL. Farmaco.* 39: 33-37, 1984.
- Madsen, H. and Ditzel, J.: Changes in Red Blood cell oxygen transport in diabetic pregnancy. *Am. J. Obstet. Gynecol.* 143:421 1982.
- Manani, G., Calzigna, L., Costa, G. and Tambascio: Clinical use of Fructose 1,6 diphosphate. *Aggressologie.* 18: 207-212, 1977.

Copyright by Mahidol University

- Manani, G., Valenti, S., Segatto, F., Giunta, F., Maritano, F., Nunziata, A., et al.: Double blind study of the treatment of the adynamic ileus with fructose 1,6 diphosphate. *Aggressologie*. 23: 67-69, 1981.
- Mansell, M. and Grimes, A.J.: Red and white cell abnormalities in chronic renal failure. *Br. J. Haematol.* 42: 169, 1979.
- Marion, I.B., Henry, R.L. and Lusher, J.M.: The Erythrocyte. *Sickle cell* ed. by H.L. Gross, pp. 15, Michigan, 1974.
- Marsili, G., Nunziata, A., Alessi, A. and Santorelli, J.: Influence of the treatment with fructose 16 diphosphate under glucose loading on blood levels of glucose, ATP, ribose, pyruvate, lactate, NEFA, glycerol, triglycerides and urea. *Clin. Terap.* 77: 65-76, 1976.
- Marsilii, A., Pata, R. and Saratti, A.: Comparative study of Fructose-1,6-Diphosphate effects on the intermittent claudication. *Agressologie*.
- Masouredis: Preservation and clinical use of blood and blood component in hematology. *Hematology*, ed. by W.J. Williams pp. 1532, McGraw-Hill, Inc, 1983.
- McMillan, D.E., Utterback, N.G., Puma, J.L. and Barbara, S.: Reduced Erythrocyte Deformability in Diabetes. *Diabetes* 27: 895-901, 1978.
- Messeter, L., Ugandu, L., Monti, M., Lundh, B., and Low, B.: CPD-adenine as a blood preservative. *Studies in vitro and in vivo. Transfusion*, 17: 210, 1977.

- Michal, G. and Beutler, H. 1974. D-Fructose-1,6-diphosphate, Dihydroxyacetone Phosphate and D-Glyceraldehyde-3-phosphate phosphate. Methods of Enzymatic Analysis, ed. by H.U. Bergmeyer, pp. 1314, Academic Press, Inc., 1974.
- Nakao, M., Nakao, T. and Yamazoe, S.: Adenosine triphosphate and Maintenance of shape of the human Red cells. Nature 187: 945-946, 1960.
- Nielsen, T.T.: Plasma citrate during submaximal and intermittent supramaximal exercise. Scand. J. Clin. Lab. Invest., 38: 29-33, 1978.
- Nielsen, T.T.: Plasma citrate in relation to glucose and free fatty acid Metabolism in man. Danish Medical Bulletin. 30: 357-378, 1983.
- Nielsen, T.T. and Sorensen, N.S.: Citrate in Plasma and Urine during total fasting. Acta. Med. Scand. 205: 303-307, 1979.
- Ogawa, Y. and Atkinson, D.E.: Interactions between Citrate and Nucleoside Triphosphates in binding to Phosphofructokinase. Biochemistry, 24: 954-958, 1985.
- Perroni, L., Ensoli, G., Nunziata, A., Soscia, F., Costrini, R., Gioia, N. and Perri, G.C.: Comparative study of the effect of Fructose-1,6-Diphosphate, fructose and physiological saline on the Blood levels of Glucose and Adenosine triphosphate after and oral glucose load. Pharm. Res. Commun. 12: 147-153, 1980.
- Pollock, A., and Cotter, K.P.: Oxygen transport in anemia. Br. J.

Haematol. 25: 631, 1973.

Prosdocimin, M., Caparrotta, L., Dorigo, P., Fassina, G. and Siliprandi, N.: Effect of Exogenous phosphorylated monosaccharides on hormone-sensitive Lipolysis in Rat adipose tissue. *Biochem. Pharmacol.* 28: 281, 1979.

Purcell, Y., and Borzovic, B.: Red cell 2,3-diphosphoglycerate concentration in man decreases with age. *Nature* 251: 511, 1974.

Ranney, H.M. and Sharma, V.S.: Oxygen equilibria of hemoglobin the oxygen dissociation curve. *Hematology*, ed. by W.J. Williams, pp. 360, McGraw-Hill, Inc, 1983.

Rigobello, M.P., Deana, R. and Galzigna, L.: Enzymatic events following the interaction of fructose 1,6 diphosphate with red cell membranes. *Advances in Pathology*, pp. 216-225, Oxford: Pergamon Press, 1982.

Roberts, A.P., Stony, C.J. and Ryall, R.G.: Erythrocyte 2,3-bisphosphoglycerate concentrations and hemoglobin glycosylation in normoxic Type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 26: 389-391, 1984.

Rose, I.A., Warms, J.V.B., and O'Connell, E.L.: Role of inorganic phosphate in stimulating the glucose utilization of human red blood cells. *Biochem Biophys. Res. Commun.* 15: 33, 1964.

Schonbein, H.S. and Volger, E.: Red cell Aggregation and Red cell deformability in Diabetes. *Diabetes* 25: 897-902, 1976.

Copyright by Mahidol University

- Shalev, O., Mogilner, S., Shinar, E., Rachmilewitz, E.A. and Schrier, S.L.: Impaired Erythrocyte Calcium Homeostasis in B-thalassaemia. *Blood* 64: 564-566, 1984.
- Sheetz, M.P., and Singer, S.J.: On the mechanism of ATP-induced shape changes in human erythrocyte membranes. The role of the spectrin complex. *J. Cell Biol.* 73: 638-646, 1977.
- Sochor, M., Gonzalez, A.M. and Mclean, P.: Regulation of alternative pathways of glucose metabolism in Rat heart in Alloxan diabetes: Changes in the pentose phosphate pathway. *Biochem. Biophys. Res. Commun.* 118: 110-116, 1984.
- Tanaka, Y. and Asakura, T.: Rapid incorporation of 2,3 diphosphoglycerate into Red Blood cells. *Lancet* 2: 571, 1984.
- Thomassen, A., Nielsen, T.T., Bagger, J.P., Charles, P., Lovgreen, N.A. and Sorensen, N.S.: Circadian Plasma Citrate Rhythms in Juvenile Diabetes. *Acta Med. Scand.* 210: 163-171, 1981.
- Tornheim, K.: Activation of muscle Phosphofructokinase by Fructose 2,6-bisphosphate and Fructose 1,6 bisphosphate is differently affected by other regulatory metabolites. *J. Biol. Chem.* 260: 7985-7989, 1985.
- Tornhiem, K. and Lowenstein, J.M.: Control of phosphofructokinase from rat skeletal muscle: Effects of fructose diphosphate, AMP, ATP, and citrate. *J. Biol. Chem.* 251: 7322-7328, 1976.
- Torrance, J., Jacobs, P., Lenfant, C., and Finch, C.A.: Intraerythrocytic adaptation to anemia. *Blood* 34: 843, 1969.
- Torrance, J., Jacobs, P., Restrepo, A., Eschbach, J., Lenfant, C.

- and Finch, C. A.: Intraerythrocytic adaptation to anemia. N. Engl. J. Med. 283: 165, 1970.
- Valentine, W.N., Oski, F.A., Paglia, D.E., Baughan, M.A., Schneider, A.S., and Naiman, J.L.: Heredity hemolytic anemia with hexokinase deficiency. Role of Hexokinase in erythrocyte aging. N. Engl. J. Med. 276: 1, 1967.
- Vora, S., Seaman, C., Durham, S., and Piomelli, S.: Isozymes of human phosphofructokinase: Identification and subunit structural characterization of a new system. Proc. Natl. Acad. Sci. U.S.A. 77: 62-66, 1980.
- Wallach, J. : Interpretation of Diagnostic Tests. A Handbook Synopsis of Laboratory Medicine, ed. by J. Wallach, Little, Brown and Company Boston, 1974.
- Wautier, J.L., Paton, R.C., Wautier, M.P., Pintigny, D., Abadie, E., Passa, P. and Caen, J.P.: Increased adhesion of erythrocytes to endothelial cells in Diabetes Mellitus and its relation to vascular complications. N. Engl. J. Med., 305: 237-242, 1981.
- Waterbury, L., and Frenkel, E.P.P.: Heredity nonspherocytic hemolysis with erythrocyte phosphofructokinase deficiency. Blood 39: 415, 1972.
- Weatherall, D.J. and Clegg, J.B.: Red cell metabolism. The Thalassaemias syndrome 3rd edition, Blackwell scientific publications, 1981, pp. 236.



001220

Weatherall, D.J. and Clegg, J.B.: Endocrine dysfunction. The

Thalassaemias syndrome 3rd edition, Blackwell scientific publications, 1981, pp. 236.

Weed, R.I.: The importance of Erythrocyte Deformability Am. J. Med. 49: 147-150, 1970.

Westhead, E.W., Kiener, P.A., Carroll, D. and Gikner, J.: Control of Oxygen Delivery from the Erythrocyte by modification of Pyruvate Kinase. Current topics in cellular regulation, 24: 21, 1984.

William, W.J., Beutler, E., Erslev, A.J. and Lichtman, M.A.: Deformability. Hematology, ed. by W. J. Williams, pp. 277, McGraw-Hill, Inc, 1983.

Winthrobe, M.M., Lee, G.R., Boggs, D.R., Bithell, T.C., Foerster, J., Athens, J.W. and Lukens, J.N.: The mature Erythrocyte. Clinical Hematology, ed. by M. M. Winthrobe, pp. 75, Lea & Febiger, Philadelphia, 1981.

Winthrobe, M.M., Lee, G.R., Boggs, D.R., Bithell, T.C., Foerster, J., Athens, J.W. and Lukens, J.N.: Erythrocyte Aging. Clinical Hematology, ed. by M. M. Winthrobe, pp. 175, Lea & Febiger, Philadelphia, 1981.

Winthrobe, M.M., Lee, G.R., Boggs, D.R., Bithell, T.C., Athens, J.W. and Foerster, J.: Normal Development of the erythrocyte. Clinical Hematology, ed. by M. M. Winthrobe, pp. 81, Lea & Febiger, Philadelphia, 1974.

Copyright by Mahidol University