STUDIES ON THE Ca^{2+}-ATPase OF THALASSEMIC RED BLOOD CELL MEMBRANES

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
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Thesis
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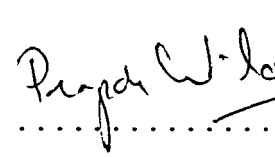

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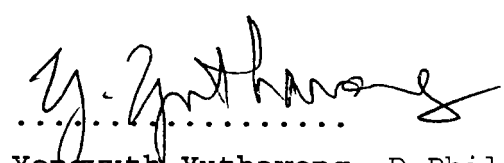

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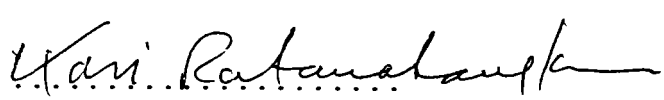

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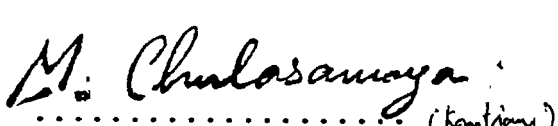

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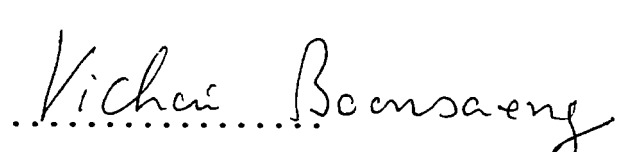

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Udompun Khansuwan

ชื่อเรื่องวิทยานิพนธ์ การศึกษาการทำงานของเอนไซม์แคลเซียม เอ ที พี เอส ที่อยู่ใน เยื่อหุ้มเซลล์เม็ดเลือดแดง ซึ่งมีพยาธิสภาพเป็นโรคธาลาสซีเมีย

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บทคัดย่อ

โรคธาลาสซีเมียเป็นโรคที่ถ่ายทอดทางพันธุกรรม มีความผิดปกติในการแตกของเซลล์เม็ดเลือดแดง สาเหตุของโรคเกิดจากมีการสังเคราะห์สายโกลบินอย่างไม่สมดุลย์ มีรายงานว่า เซลล์เม็ดเลือดแดงที่เป็นธาลาสซีเมีย มีแคลเซียมอิออนสะสมอยู่ปริมาณมาก ในเซลล์เม็ดเลือดแดงของคนปกติมีความเข้มข้นของแคลเซียมอิออนต่ำมาก ซึ่งการควบคุมทำได้โดย 2 กลไก คือ 1. คุณสมบัติของเยื่อหุ้มเซลล์ในการเลือกจำกัดการนำแคลเซียมอิออนเข้าสู่เซลล์ และ 2. การขับแคลเซียมอิออนออกจากเซลล์โดยการทำงานอย่างมีประสิทธิภาพของเอนไซม์ แคลเซียม เอ ที พี เอส ซึ่งฝังตัวอยู่กับเยื่อหุ้มเซลล์

ความผิดปกติของเอนไซม์ เอ ที พี เอส ที่ทำหน้าที่ปั๊มแคลเซียมแสดงให้เห็นได้โดยการศึกษา คุณสมบัติอาร์เรเนียส, การตอบสนองต่อการกระตุ้น

ของคาลโมดูลลิน และ ความเข้มข้นของแคลเซียมอิออน และ การเกิดสารตัวกลางฟอสโฟเอนไซม์ พบว่า แคลเซียม เอ ที พี เอส จากเซลล์เม็ดเลือดแดงธาลาสซีเมียทุกชนิดสูญเสียการตอบสนองต่อคาลโมดูลลิน คุณสมบัติอาร์เรเนียสเปลี่ยนไป กล่าวคือ จุดหักอาร์เรเนียสที่ 32 °ซ หายไป และ ค่าพลังงานของการกระตุ้น ปฏิกิริยาลดลง และ ปั๊มแคลเซียมที่ได้จากธาลาสซีเมียทุกชนิด สูญเสียความสามารถในการเกิดฟอสโฟเอนไซม์อีกด้วย ค่าสังเกตเหล่านี้แสดงให้เห็นว่า การทำงานของ เอ ที พี เอส และ การขนส่งแคลเซียม ซึ่งอยู่ต่างตำแหน่งกันบนโมเลกุลของปั๊มแคลเซียม ได้ถูกแยกขาดออกจากกัน

สาเหตุที่ทำให้เกิดความเสียหายแก่ แคลเซียม เอ ที พี เอส ในธาลาสซีเมีย ได้สืบสวนไปยังผลการเกิดภาวะออกซิเดชั่นโดยอนุมูลอิสระของออกซิเจน จึงได้นำเอาเซลล์เม็ดเลือดแดงปกติ มาทำปฏิกิริยากับสารออกซิเดชั่นหลายชนิด ได้แก่ เฟนนาซีน เมโทซัลเฟต เฟนนิลไฮดราซีน เทอร์เชียรีบิวทิลไฮโดรเปอร์ออกไซด์ และ แซนธีน ออกซิเดส แล้วจึงศึกษา คุณสมบัติอาร์เรเนียส การตอบสนองต่อคาร์โมดูลลิน และแคลเซียม ผลการทดลองแสดงให้เห็นว่าเอนไซม์จากเม็ดเลือดแดงที่ถูกออกซิไดส์ ด้วยอนุมูลอิสระของออกซิเจนมีการเปลี่ยนคุณสมบัติต่างๆดังกล่าว ไปในลักษณะที่คล้ายกับที่พบในโรคธาลาสซีเมีย

นอกจากนี้จาการทดลองยังได้พบว่าอนุมูลอิสระของออกซิเจนมีผลเพิ่มการนำแคลเซียมเข้าสู่เม็ดเลือดแดงอีกด้วย

ในการศึกษานี้ จึงสรุปว่า การสะสมแคลเซียม ในเซลล์เม็ดเลือดแดงธาลาสซีเมียสามารถเกิดขึ้นได้ ทั้งจากการยับยั้งการทำงานของ แคลเซียม เอ ที พี เอส และการเพิ่มการผ่านของแคลเซียมเข้าเซลล์ ซึ่งเป็นผลสืบเนื่องมาจากมีการผลิตอนุมูลอิสระของออกซิเจนขึ้นมาภายในเซลล์อย่างมากมาย ภายหลังจากที่ฮีโมโกลบินตกตะกอนจับบนเยื่อหุ้มเซลล์เม็ดเลือดแดง

Thesis Title Studies on the Ca^{2+}- ATPase of Thalassemic Red Blood Cell Membranes

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ABSTRACT

Thalassemia is a genetically inherited hemolytic disorder caused by imbalance synthesis of the globin chains. Accumulation of calcium ions has been reported in the thalassemic erythrocytes. Regulation of very low concentration of Ca^{2+} ions in normal human erythrocytes is accomplished by two mechanisms: slow inwards transport of the Ca^{2+} ions by selective permeability of the membrane and high efficiency protrusion of the Ca^{2+} ions by the membrane-bound Ca^{2+}-ATPase.

The abnormalities of the Ca^{2+} pump-ATPase have been elucidated by study of Arrhenius curve , response to calmodulin stimulation, Ca^{2+}-dependency, and the formation of phosphoenzyme intermediate. The Ca^{2+}-ATPase of erythrocytes from all types of thalassemias showed loss of calmodulin response, changes in the Arrhenius properties with the disappearance of the discontinuity at 32°C, a decrease in the value of activation energy at

temperture lower than 32°C, and an increase in the Ca^{2+}/calmodulin-independent ATPase activity. The Ca^{2+} pump-ATPase from all types of thalassemias showed loss in the ability to form phosphoenzyme intermediate. These observations suggest an uncoupling of the two activities, ATPase and Ca^{2+}-translocation, which locate on different active centers on the plasma membrane Ca^{2+} pump.

The cause of the damages to the thalassemic Ca^{2+}- ATPase was investigated by examining the oxidative effects of oxygen free-radicals. Normal erythrocytes were treated with various oxidants (phenazine methosulphate, phenylhydrazine, tert-butylhydroperoxide and xanthine oxidase) and the Arrhenius properties, calmodulin response, and Ca^{2+}-dependency were examined. The results showed that the changes in the properties of the enzyme from oxygen free-radical treated erythrocytes were very similar to those observed in thalassemic erythrocytes.

In addition, the effects of oxygen-free radicals to increase in Ca^{2+}-permeability have also been observed in experiments on human erythrocytes.

It was concluded in this study that an accumulation of Ca^{2+}ions in thalassemic erythrocytes could caused by both uncoupling of the Ca^{2+} pump and increase in Ca^{2+}-permeability. The effects could be the consequences of intracellular overproduction of oxygen free-radicals following precipitation of hemoglobin onto the erythrocyte plasma membrane.

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ABBREVIATIONS

Ca^{2+}	= Calcium ion
°C	= Degree Celcius
Cu	= Copper
Fe	= Iron
g	= Gram
xg	= Times of the gravitational force
Hb	= hemoglobin
kDa	= Kilo Dalton
L	= Liter
µg	= Microgram
µl	= Microliter
µmole	= Micromole
µM	= Micromolar
mg	= Milligram
ml	= Milliliter
mM	= Millmolar
min	= Minute
M	= Molar
nmole	= Nanomole
%	= Percent
P	= Phosphorus
PCV	= Packed Cell Volume
RBC	= Red Blood Cell
rpm	= Revolution per minute
SDS-PAGE	= Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
V/V	= Volume/volume

CHAPTER 1

INTRODUCTION

1.1 Erythrocyte

The erythrocyte, commonly called the red blood cell (RBC), is the cell in the circulation whose functions is to bind, transport and deliver oxygen and carbon dioxide between lung and other tissues. The cells delvelope from undifferentiated, pluripotential stem cells. Following multiple cell divisions, a subset of these pluripontential cells becomes committed to the erythroid lineage; others retain their pluripontency and maintain the stem cell pool. Following commitment, erythroid progenitors progress through several replication stages, becoming more functionally specialized with maturation. Eventually, the reticulocyte and finally the mature, circulating erythrocyte are produced (Figure 1).

Collectively, the erythroid progenitors and adult red cells have been termed the erythron to reinforce the idea that they function as an organ (1). The erythron has three cell compartments. The most primitive is the pool of early erythroid progenitors that are characterized by their capability to give rise to erythroid colonies *in vitro*. An intermediate cell population is made up of the erythroid precursors from proerythroblasts to marrow reticulocytes. The last one is the circulating mature erythrocytes.

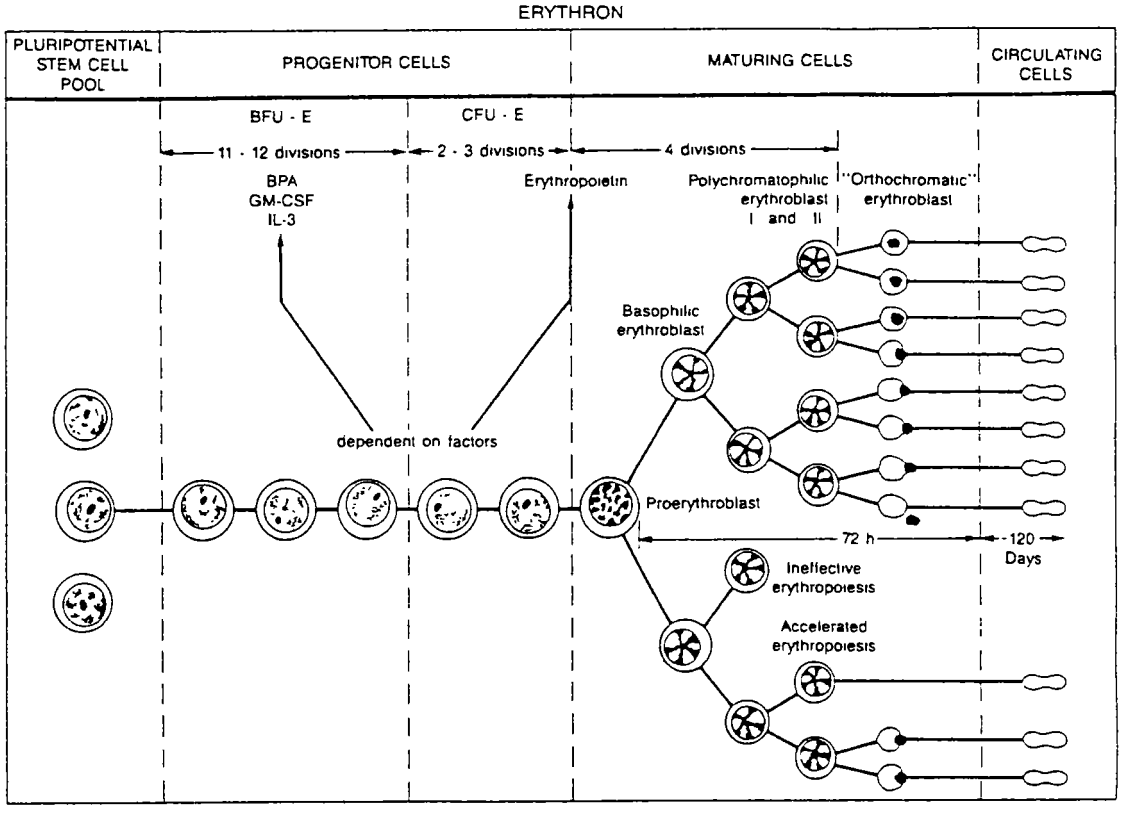


Figure 1: The Erythron. The erythropoietic organ and circulating red cell mass constitute the erythron. Depicted here are the composition of the erythron and the maturation and amplification of the red cell series. Pluripotential stem cells committed to the erythrocyte undergo to the erythroid lineage, progressing through replicative progenitor stages known as burst forming unit-erythroid (BFU-E) and then colony forming unit-erythroid (CFU-E) stages before reaching the distinctive erythroblast stages of terminal maturation. The proerythroblast matures to a reticulocyte through four arbitrarily defined stages: the basophilic erythroblast, polychromatophilic erythroblasts I and II, and the orthochromatic erythroblast, which, by ejection of its nucleus, emerges as a reticulocyte. The maturation process requires a period of approximately 72 h; in the ensuing 48 h, the circulating reticulocyte matures into an erythrocyte. (From reference 1)

The erythrocyte contains no nucleus and other intracellular organelles and no detectable RNA and DNA. The shape is biconcave disk with a diameter varying from 6 to 9 microns and a thickness of about 1 micron at the centre, increasing to 2 to 2.5 microns towards the periphery. The prime role of the erythrocyte in the delivery of oxygen and carbon dioxide is accomplished by the presence of a 74 % solution of hemoglobin (Hb). The cell contains the most concentrated protein solution of about 15 g of Hb per 100 ml of whole blood without the high viscostiy that would attend the equivalent amount of protein in free solution. The details of the composition of the erythrocyte have been summarized by Ernest Beutler (2). The electrolyte composition of the erythrocyte is similar to cells generally. The chief cation is K^+, with lesser amounts of Na^+, Ca^{2+},and Mg^{2+}, and the major anions are Cl^-, HCO_3^-, Hb,and inorganic phosphate. 2,3-diphosphoglycerate is the major phosphate-containing component (Figure 2). The internal composition of the erythrocyte is maintained by energy requiring mechanisms. The erythrocyte membrane shows several ATPase activities, e.g. Mg^{2+}-dependent Ca^{2+}/H^+ATPase, Mg^{2+} - ATPase and Na^+/ K^+- ATPase, which influence the transport of the specified cations across cell membrane.

The life-span of normal erythrocyte is approximately 120 days. Since the erythrocyte loses intracellular organelles before entering the circulation,it does not have the capacity to synthesize new enzyme molecules to replace those that may become worn out during its life-span. The enzymes present in

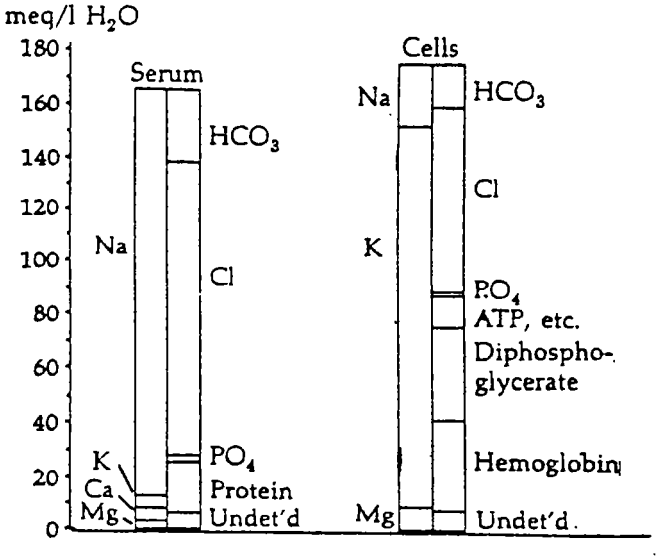


Figure 2: The electrolyte structure of the oxygenated human erythrocyte. (From reference 4)

erythrocyte are formed largely by the nucleated marrow cells and , to a lesser extent, the reticulocyte. For survival, the erythrocyte must have a source of energy. This energy is needed to maintain (1) the iron of hemoglobin in the divalent form, (2) the high potassium and low calcium and sodium levels within the cell against a gradient imposed by high plasma calcium and sodium and low plasma potassium levels, (3) the sulfhydryl groups of red cell enzymes and of hemoglobin in the active, reduced forms, and (4) the biconcave shape of the cell. If the erythrocyte is deprived of a source of energy, it becomes sodium- and calcium- loaded and potassium depleted and the shape changes from a biconcave disc to a sphere. Such a cell is rapidly removed from the circulation by the filterring action of the spleen and liver. Even if it survives, such an energy-deprived cell would gradually turn brown as hemoglobin is oxidised to methemoglobin by the very high concentration of oxygen within the erythrocyte. The cell would then be unable to perform its function of oxygen and carbon dioxide transport. The erythrocyte can utilize energy solely from glucose, and thus the cell depends on the only one ATP-generating metabolic pathway, the anaerobic glycolysis.

1.2 **Hemoglobin**

Hemoglobin is the major protein component of the erythrocyte. Normal concentrations of hemoglobin in adult human erythrocyte vary between 14.0 - 17.5 g/dl blood in men and 12.3 - 15.3 g/dl blood in women. Its prime function is to bind and to deliver oxygen and

carbon dioxide between lung and other tissues. The hemoglobin molecule composes of the protein moiety called globin and the red chromophore named heme or ferrous protoporphyrin IX.

1.2.1 **Heme**

Heme is essential for the function of all aerobic cells. It serves as the prosthetic group of hemoglobin and other enzymes, e.g. cytochromes, catalase, peroxidase and tryptophan pyrolase. The structure of heme is shown in Figure 3a. It composes of an iron atom coordinated to the four pyrole rings of the porphyrin through the nitrogen atom in each pyrole ring. The β-substituent position of heme, that is, the outer periphery of the porphyrin macrocycle, is fully substituted with alkyl groups. Substitution for the methene bridge carbon atoms does not occur in heme.

Furrous ions have six electron pairs per atom. Thus the ferrous iron atom in heme, bound to the pyrole nitrogen via four electron pairs, has still two other unoccupied electron pairs, one above and one below the plane of the porphyrin ring. In hemoglobin, one of these pairs is coordinated with a histidyl resdue of the globin chain (Figure 3b). This histidine is an invariable feature of all normal vertebrate globin chains. The other coordination site of iron is opened in deoxyhemoglobin and protected from oxidation by nonpolar environment provided by the amino acid residues surrounding the heme moiety. It is the sixth coordination position of the iron atom in hemoglobin which binds the oxygen molecule for transport.

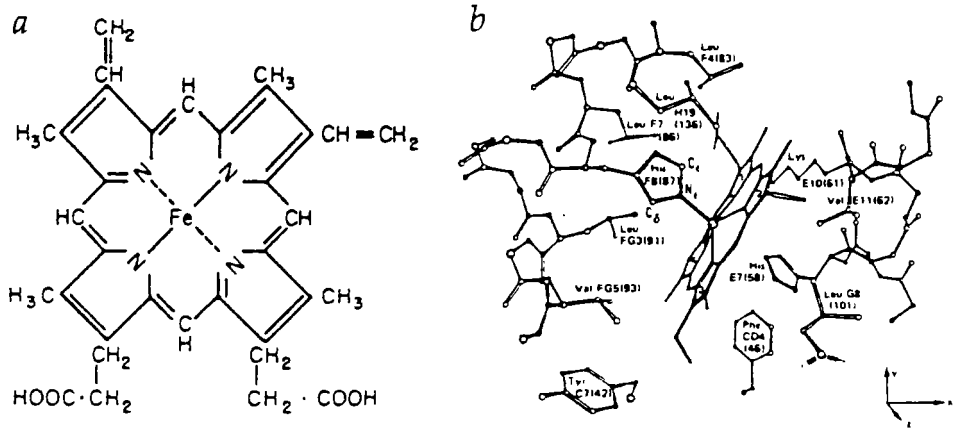


Figure 3: (a) Structure of heme (ferroprotoporphyrin IX). (b) Heme group and its environment in the unliganded chain. Only selected side chains are shown : the heme 4-propionate is omitted. (From reference 6)

The iron in hemoglobin must be in the ferrous state in order to be able to bind oxygen reversibly. If the iron is oxidised to ferric form, hemoglobin is no longer capable of binding oxygen. Although oxidised hemoglobin, that is methemoglobin, is generated in erythrocyte, it is continuously reduced to ferrous hemoglobin in the cell by the NADH-cytochrome b_5 reductase/ cytochrome b_5 system. When heme is released from the globin moiety, it is immediately oxidised to hemin.

Approximately 85 % of body heme is synthesized in the erythropoietic marrow, and the remainder is synthesized largely by the liver. In the liver, the majority of heme synthesized is incorporated into microsomal cytochromes P_{450} which performs important biotransformation of a variety of chemicals: carcinogens, steroids, vitamins, fatty acids and prostaglandins. The biosythetic pathway requires eight consecutive enzymatic reactions and occurs within two cellular compartments, mitochondria and cytoplasm (3,4,5,6). The rate-limiting enzyme is the δ-aminolevulinic acid (ALA) synthase [Succinyl CoA : Glycine C-Succinyl transferase (decarboxylating) (E.C. 2.3.1.37)] which is localized in the inner membrane of mitochondria. The enzyme activity is feed-back controlled by heme, the end product of the biosynthetic pathway. Regulation of ALA synthase level by heme occurs at at least four different levels, that is, (1) transcription,(2) translation,(3) transfer into mitochondria, and (4) enzyme inhibition. The last mechanism appears the least important since relatively high concentration of hemin is necessary for the

inhibition of the enzyme activity and the amount of heme generated in mitochondria is not sufficient to achieve such a concentration. In contrast, heme exerts significant inhibitory effects on ALA synthase at the level of transcription, translation and transport.

1.2.2 **Globin**

The biosynthesis of human globin involves the usual pathway of information flow in eucaryotic cells from DNA to RNA to protein (Figure 4). The globin genes are organized in a linear array on chromosomes 11 and 16 (Figure 5). The ε-,γ-, δ-, and β- genes which are collectively called the β-gene cluster, are all linked on a single piece of DNA on chromosome 11. The δ- and β-genes are used in the biosynthesis of adult hemoglobin (hemoglobin A, $\alpha_2\beta_2$; hemoglobin A_2, $\alpha_2\beta_2$) and are about 5.5 kilobases (kb) apart at the 3'end of the complex. The two γ -genes, $^{G}\gamma$ and $^{A}\gamma$ are separated from each other by about 3.5 kb, and the $^{A}\gamma$-gene is about 15 kb 5' to the δ -gene. The ε-gene is 5' to the gene (7).

The α-genes are linked on a single DNA fragment on chromosome 16. Two α -genes (α_1 and α_2) are separated by about 2.5 kb. Two embryonic ζ-genes are located 5' to the α-genes. One of these ζ-genes is active, while other is inactive. In the β-gene complex, there is one so-called pseudogene, a sequence of DNA that does not contain all the structural features required for a functionally intact globin gene but that has significant nucleotide homology with the β-like

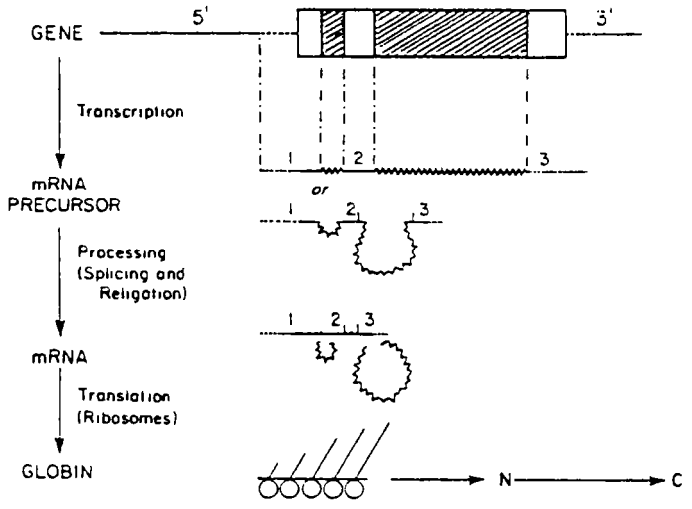


Figure 4: Globin biosynthesis. The β-globin gene coding sequences (clear areas of rectangle) are interrupted by two intervening sequences (hatched areas). Globin mRNA precursors include intervening sequences (~~~~~) which are removed by splicing and religation. (From reference 7)

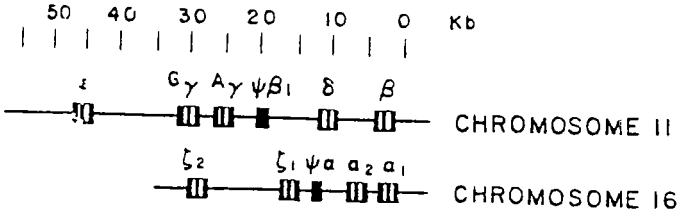


Figure 5: Organization of human globin genes. The ε, γ, δ, and β genes are on chromosome 16. The distance between the genes is shown in kilobases (kb) at the top. The black regions within each rectangle are coding regions of the genes, and the clear regions the intervening sequences.

genes. The pseudogene (pseudo β_1) is located between the $^{A}\gamma$- and δ- genes (Figure 4). A pseudo α- gene is located between the ζ- and α- genes (7).

The globin gene complex also contains so-called inverted repeat sequences in specific regions flanking the structural genes. These sequences are located 5' to the ε-, $^{G}\gamma$-, and δ- genes, and 3' to the $^{A}\gamma$- and β-genes, and are about 250 base-pairs (bp) long. They occur in regions which may be marked as specific domains of globin gene transcription, since they are at the boundaries of ε, γ, δ and β transcriptional complexes. These inverted repeats, such as 5' CCTT.....AAGG 3', can form intramolecular double-stranded structures because of their complementary sequences. The structure can initiate specific RNA transcription in the presence of RNA polymerase III (7).

Each globin gene contains two introns (Figure 6). A small intron (intron 1) located between codons 30 and 31 of all non α-genes, that is ε-, γ-, δ- and β-genes, and a large intron (intron 2) between codons 104 and 105 in each of these genes. The location of introns 1 and 2 in the α-and ζ-genes is different only because of a reduction in the total number of codons from 146 to 141 in these genes as compared with the non α-genes. At the 3' end of the structural gene, there are extra 75 to 100 untranslated (noncoding) nucleotides which include the sequence AATAAA, identifying the site at which polyadenylation of mRNA occurs (7).

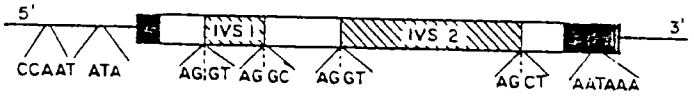


Figure 6: β-Globin gene structure. The β-globin gene coding sequences (clear areas) are interrupted by two intervening sequences (hatched areas). The black areas are the 5′ and 3′ flanking regions. The nucleotide sequences shown are the largely conserved sequences in the 5′ and 3′ junctions of IVS 1 and IVS2, in the 3′ untranslated region, and in the 5′ flanking region. (From reference 7)

Two regions 5' to the gene important for globin gene transcription are the so-called TATA and CAAT box sequences, also found in almost all other eucaryotic genes studied. The TATA or ATA sequences are about 30 to 40 bp 5'to the cap site, while the CAAT box sequences are about 70 to 80 bp 5' to this site (Figure 6). These regions are involved in RNA polymerase binding and interactions between globin genes and a variety of other nuclear proteins. Sequences further 5' to the CAAT sequences are also implicated in the regulation of β- and γ-globin genes and also participate in interactions with nuclear proteins. These elements are defined as promotors and enhancers. Promotors are sequences 5' to the gene that are critical for the interaction of specific genes with RNA polymerase II, which leads to optimal transcription of these genes into RNA. Enhancers are elements that increase RNA transcription and may be indistinguishable from promotors at first analysis. Enhancers, however, have the additional properties of being active DNA fragments when placed in either 5′ or 3′ orientation and whether located upstream, downstream or within specific genes. These enhancers sequences presumably function by interacting with tisue-specific protein factors to alter the structure of RNA transcription complexes to enhance promotor function and gene transcription. These are β-gene enhancers present 3′ to the chick and human β-globin genes, and γ-gene enhancers present in the 3′ region flanking the human γ-gene (7).

Globin gene transcription begins at the cap sequence 5' to the gene and proceeds through the

5' untranslates region and through nucleotides used in coding for amino acids (coding sequences or exons) and intervening sequences (IVS or introns). Polyadenylation of the mRNA takes place in the nucleus rapidly after RNA transcription. The cap site, at which transcription begins, is methylated and contains no free 5' phosphate. The structure of the cap site is 7-methyl, 5',5'-guanine, 6-methyl adenosine. After capping and adenylation, introns are spliced from the RNA precursors, leaving an abundance of long-lived mature mRNA in the cytoplasm. The message becomes associated with polyribosomes, and in the presence of activating enzymes, initiation, elongation and termination factors, amino acids and tRNAs, globin mRNA translation produces the mature globin product. Heme stimulates globin mRNA translation (7). Intact hemoglobin tetramers are formed by addition of heme to the globin subunits after their syntheses and release from polyribosomes (Figure 4). As far as is known, the synthesis of α- and β-globin occurs independently on polyribosomes, and the assembly of hemoglobin is directed solely by the physical and chemical affinities of globin subunits, and their interaction with heme (7).

1.2.3 **Structure of Hemoglobin**

Human hemoglobin is a tetramer composed of two α-like and two non α-chains associated together by noncovalent interactions as salt bridges, hydrophobic contacts and hydrogen bonds (Figure 7). The α-chain is 141 amino acids in length. The N-terminal is valyl-

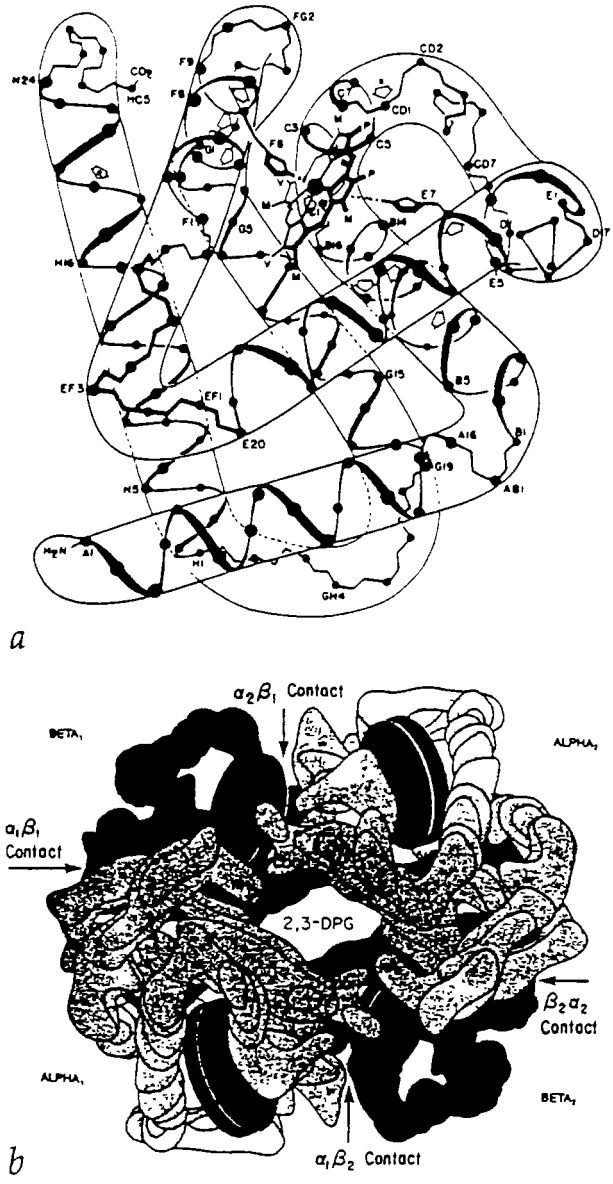


Figure 7: (a) The representation of the structure of globin chains. Arrows indicate sites of substitutions in a number of unstable hemoglobins. (b) The hemoglobin molecule, as deduced from x-ray diffraction studies, shown from above. The molecule is composed of four subunits: two identical α chains (light blocks) and two identical β chains (dark blocks). 2,3-DPG binds to the two chains in the deoxyhemoglobin molecule. (From reference 7)

leucine, and the C-terminal is tyrosyl-arginine. The α- and ζ-chains lack the residues of the D helix of the β-chain and are shorter by five residues than the β-chain. The β-chain is 146 amino acids in lenght, beginning with valyl-histidine at the N-terminus and ending with tyrosyl-histidine at the C-terminus.

Throughout the course of a human lifetime, 6 different normal hemoglobins are sequentially synthesized, and can be divided into 3 categories as embryonic, fetal and adult hemoglobins (Table 1). During the human development from embryo to early infant, the sequences of both α-like and non α-like globins is exactly the same:in the order from 5' to 3' of the globin gene clusters (Figure 8). Hb Gower$_1$ ($\zeta_2\varepsilon_2$) is the major hemoglobin of embryos of less than 5 to 6 weeks of gestation. Hb Gower$_2$ ($\alpha_2\varepsilon_2$) and Hb Portland ($\zeta_2\gamma_2$) are found in embryos with gestational age as young as 4 weeks and is absent in embryos older than 13 weeks. Synthesis of ζ- and ε-chains decreases as that of α-and γ- chains increases. This progression occurs about the time that the liver replaces the yolk sac as the main site of erythropoiesis (8).

Fetal hemoglobin, HbF ($\alpha_2\gamma_2$), is present in very young embryos and is the major hemoglobin of fetal life. It constitutes 90 to 95 % of total hemoglobin in the fetus until about 34 to 36 weeks of gestation.

Synthesis of hemoglobin A ($\alpha_2\beta_2$) begins since 9 weeks of fetal life. The amount of HbA increases from 4

Table 1: Embryonic hemoglobins

Hemoglobin	*Chain composition*	*Primary site*	*Appearance*
Gower 1	$\zeta_2\varepsilon_2$	Yolk sac	<5-6 weeks
Gower 2	$\alpha_2\varepsilon_2$	Yolksac	4-13 weeks
Portland	$\zeta_2\gamma_2$	Yolksac	4-13 weeks
Fetal, F	$\alpha_2\gamma_2$	Liver	Early, 53-95% at term
Adult, A	$\alpha_2\beta_2$	Marrow	9 weeks, 5-5% at term
Adult, A2	$\alpha_2\delta_2$	Marrow	35 weeks

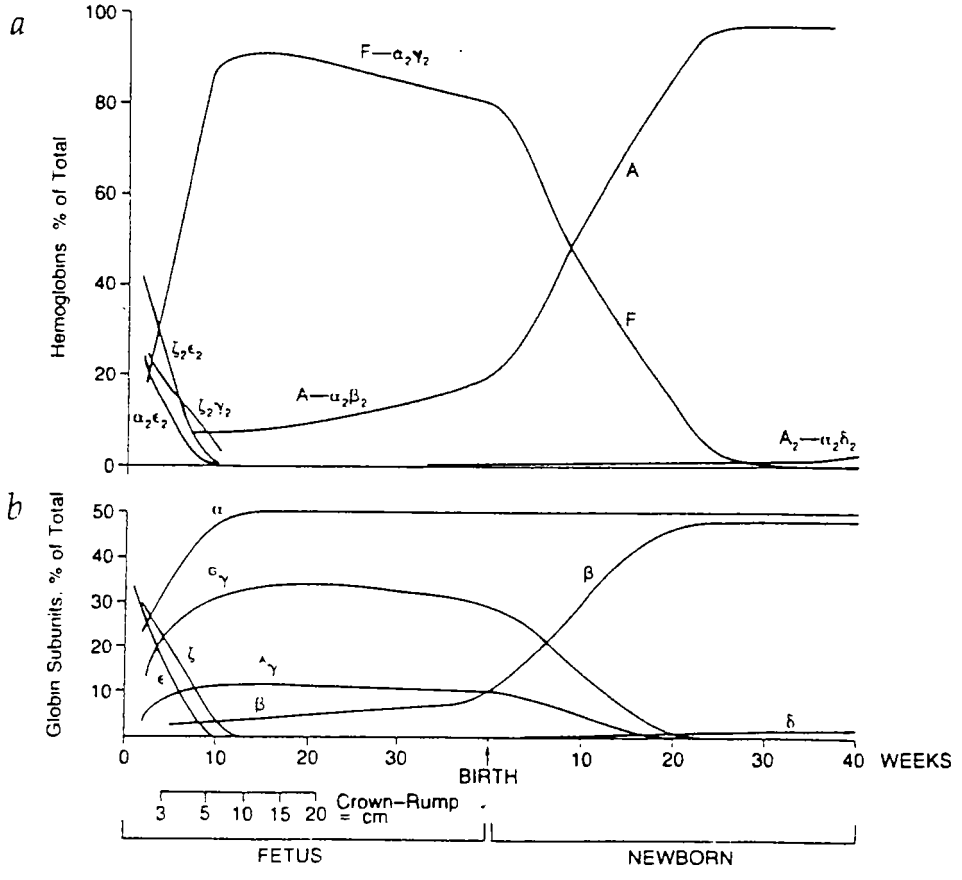


Figure 8: Changes in hemoglobin tetramers (a) and in globin subunits (b) during human development from embryo to early infancy. (From reference 8)

to 13 % when the fetal age is 21 weeks of gestation. After 36 weeks of gestation HbF decreases, and the percentage of HbA rises further. After birth, the concentration of HbF is generally less than 2 - 3 % of total hemoglobin by 6 months of age. In an adult, HbA constitutes about 97 % of the hemoglobin in the erythrocyte whereas the minor hemoglobin fractions, HbA_2 ($\alpha_2\delta_2$) and HbF are present at about 2.5 and 0.5 %, respectively (8).

1.2.4 **Abnormal Hemoglobin**

At present, hundreds of abnormal hemoglobin caused by mutation at specific sites of codons in α- and β-globin genes have been reported (For reviewing see ref 6). Among these, only hemoglobins S, C, D, E, and Constant Spring are polymorphic, whereas others are rare.

Sickle hemoglobin (HbS) composes of normal globin and variant which glutamate residue number 6 is replaced by valine ($\beta^{6glu->val}$). Molecules of deoxyHbS have a strong tendency to aggregate resulting in the formation of long HbS fiber which causes the erythrocyte to undergo sickling. HbS occurs with the greatest prevalence in tropical Africa where malaria has been common.

In hemoglobin C (HbC), the glutamate residue number 6 in β-globin is substituted by lysine ($\beta^{6glu->lys}$). Red cells containing principally HbC are

more rigid than normal, and their fragmentation in the circulation may result in the formation of microspherocytes. Erythrocyte life-span is shortened to a mean of 30 - 35 days. Erythrocytes from patients with HbC disease have a low oxygen affinity. HbC is found in 17 to 28 % among the West Africans.

Hemoglobin E (HbE) is characterized by substitution of glutamate residue number 26 of β-globin to lysine ($\beta^{26glu->lys}$). HbE is unstable when subjected to oxidative stress. Moreover, the mutation also created a new potential splicing sequence so that some of the mRNA may be spliced improperly. The formation of improper messenger accounts for the thalassemia-like nature of HbE trait and disease. HbE disease is principally found in Myanmar, Thailand, Laos, Cambodia, Malaysia and Indonesia, but is not prevalent among the Chinese. Clinical features of homozygous HbE disease are microcytosis and hypochromia but little or no anemia. Splenomegaly is unusual, and the red cell life-span is normal, the state closely resemble to β -thalassemia minor. The clinical manifestations of the heterozygous state between HbE and β-thalassemia are somewhat more severe than homozygous HbE disease, with moderate anemia and splenomegaly representing the usual manifestation.

Hemoglobin Constant Spring (Hb CS) is a hemoglobin variant with an elongated α-globin chain. The abnormality is due to a single substitution at the terminator codon TAA with CAA of the α_2-globin gene,

leading to the translation of extra 31 amino acid residues (9). The α^{CS} variant is the most prevalent nondeletion α-thalassemia in Southeast Asian populations, particularly in Laos (10). The gene frequency for α^{CS} in Thai have been reported to be about 0.008(11). Because of the instability of the α^{CS} mRNA, the abnormality can give rise to clinical symptom of thalassemia syndrome when interacting with α-$thal_1$, called Hb H disease with Hb CS (α-$thal_1$/Hb CS) or in the homozygous state (Hb CS/Hb CS) which are especially common in Thailand (12).

Only some abnormal hemoglobins are of clinical importance; they are unstable, produce erythrocytosis, or cyanosis. Many of the others do not produce significant clinical alterations but have, nontheless, been important in clarifying the role of amino acid substitution at specific sites on the structure and function of the hemoglobin molecule.

1.3 Erythrocyte Membrane

1.3.1 General Charactics of Erythrocyte Membrane

The human erythrocyte is enclosed by plasma membrane which constitutes a cell dimension of 8.5 ± 0.41 in diameter, 2.4 ± 0.13 in the greatest thickness and 1.0 ± 0.08 in the least thickness. Its surface area is about 163 μ^2 and its volume is about 80 μ^3 (13). Approximately 52 % of the membrane mass is protein, 40 %

is lipid, and 8 % is carbohydrate. Only about 7 % of the sugars is carried by glycosphingolipids and the rest takes the form of small oligosaccharides linked via O- and N- glycosidic bonds to serine or threonine and asparagine residues in the membrane glycoproteins (14).

The membrane, which surrounds the erythrocyte, forms a boundary between the interior of the cell, containing its highly concentrated solution of hemoglobin and the plasma surrounding it. The membrane serves as a barrier to help maintain in the interior of the erythrocyte a concentrations of various ions and metabolites which differ markedly from the concentrations found in the exterior environment. The membrane is insoluble in aqueous solution. Approximately one-half of the mass of the human erythrocyte membrane consists of lipids arranged as a bilayer.

In addition to serving as a barrier, the membrane contains pumps and channels for the movement of sodium, potassium, calcium and oxidised glutathione, and it facilitates the transport of glucose and other small molecules.

Normal human erythrocytes at rest are typically in a biconcave disc conformation and are named "discocytes". The cells are highly deformable. Maintenance of normal deformability is essential for the cells to successfully negotiate the small passageways in the microcirculation, particularly along

splenic sinuses where erythrocytes must squeeze through interendothelial slits that seldom exceed 0.2 - 0.5 μ in width. The remarkable durability and reversible deformability character of the cell are largely dependent upon the membrane-associated cytoskeleton, which is a scaffold of filamentous meshwork of proteins located immediately underneath the lipid bilayer (13).

1.3.2 **Membrane Lipids**

Phospholipids and nonesterified cholesterol account for more than 95 % of the total lipids within the erythrocyte membrane. Small amounts of glycolipids, glycerides and free fatty acids are also present. On a molar basis, phospholipids and cholesterol are present in nearly equal amounts. The presence of cholesterol is extremely important because its orientation within the bilayer affects the membrane fluidity and its interaction with the phospholipids stabilizes the bilayer (15).

The major phospholipids and their approximated concentrations in human erythrocytes are as follows; phosphatidylcholine, 30 %; phosphatidylethanolamine, 28 %; phosphatidylserine, 14 %; and sphingomyelin, 25 %. Small but perhaps physiologically significant amounts of phosphatidylinositides, phosphatidic acid, and polyglycerolphosphatides are also present in the red cells (16).

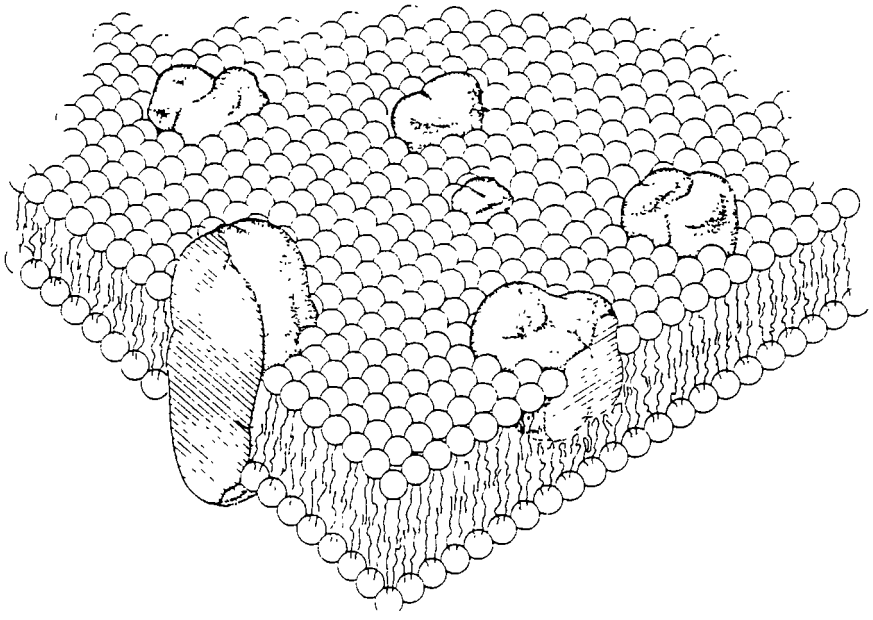


Figure 9: A schematic representation of the Singer-Nicholson fluid mosaic model for the structure of cell membranes. Irregular proteins are seen penetrating both into and through the biomolecular leaflet composed of regular arrays of phospholipid molecules. The head groups of the phospholipids face the cytoplasmic and plasma environments, while their acyl tails are enmeshed to form the lipophilic membrane core. The outer leaflet of the bilayer is composed predominantly of choline-containing phospholipids, and inner leaflet is predominantly composed of acidic phospholipids, such as phosphatidylethanolamine and phosphatidylserine. Cholesterol is shown embedded symmetrically in each leaflet among the fatty acid side groups of the phospholipids. (From reference 12)

Most of the red cell lipids is arrayed in the form of a bimolecular leaflet in which the polar head groups of each lipid layer face away from the center of the membrane into the hydrophilic environments of the cytoplasm and the plasma, while the long acyl tails of the lipids form a central hydrophobic core of the membrane . Many of the membrane proteins are inserted into this lipid matrix in much the same way as icebergs float in the ocean (Figure 9) (17, 18).

The lipids are not symmetrically distributed between the inner and outer leaflets of the erythrocyte membrane. The majority of the phosphatidylethanolamine (PE), the phosphatidylserine (PS) and the phosphatidylinositol (PI) are contained within the inner or cytoplasmic leaflet of the membrane, while the majority of the phosphatidylcholine (PC), the sphingomyelin (SM), cholesterol and the glycolipids are contained in the leaflet facing the plasma (19). The biochemical basis of this asymmetry may be the combined results of several effects, i.e. the site specificity of the phospholipid exchange and renewal reactions (16), the specificity of the ATP-dependent flippase for the aminophospholipids (20, 21), and the selective interactions between aminophospholipids and the specific cytoskeletal proteins such as spectrin (22, 23, 24) and protein 4.1 (25, 26).

1.3.3 Membrane Proteins and Membrane Skeleton

Major membrane proteins in human erythrocyte membranes are classified in two groups: integral proteins which span the bilayer, and peripheral proteins which lie immediately underneath the cytoplasmic surface of the bilayer (27). When erythrocyte membranes are dissolved in an excess of sodium dodecylsulfate (SDS), the polypeptides are denatured and separated from the lipids and from each other, as demonstrated by SDS-polyacrylamide gel electrophoresis. The majority of the membrane polypeptides and glycoproteins are named after being made visible by Coomassie blue and periodic acid Shiff (PAS) staining techniques, respectively. Some characteristic properties of major proteins in human erythrocyte membranes are illustrated in Figure 10.

Some of these proteins are reponsible for the elastic property of the erythrocyte membrane and are referred to as the cytoskeleton or membrane skeleton. The membrane skeleton is defined as the protein frame work remaining after erythrocyte membranes are extracted with nonionic detergents such as Triton X-100 at low ionic strength (28). These detergents dissolve the lipid bilayer and integral proteins which are not linked to the underlying membrane skeleton. The proteins remaining in the membrane skeleton vary with the conditions used in its preparation but includes spectrin, actin, protein 4.1, 4.9, and a few other proteins as ankyrin, and tropomyosin . The membrane skeleton can be viewed as being a meshwork of spectrin filaments which are associated with each other through three major linkages

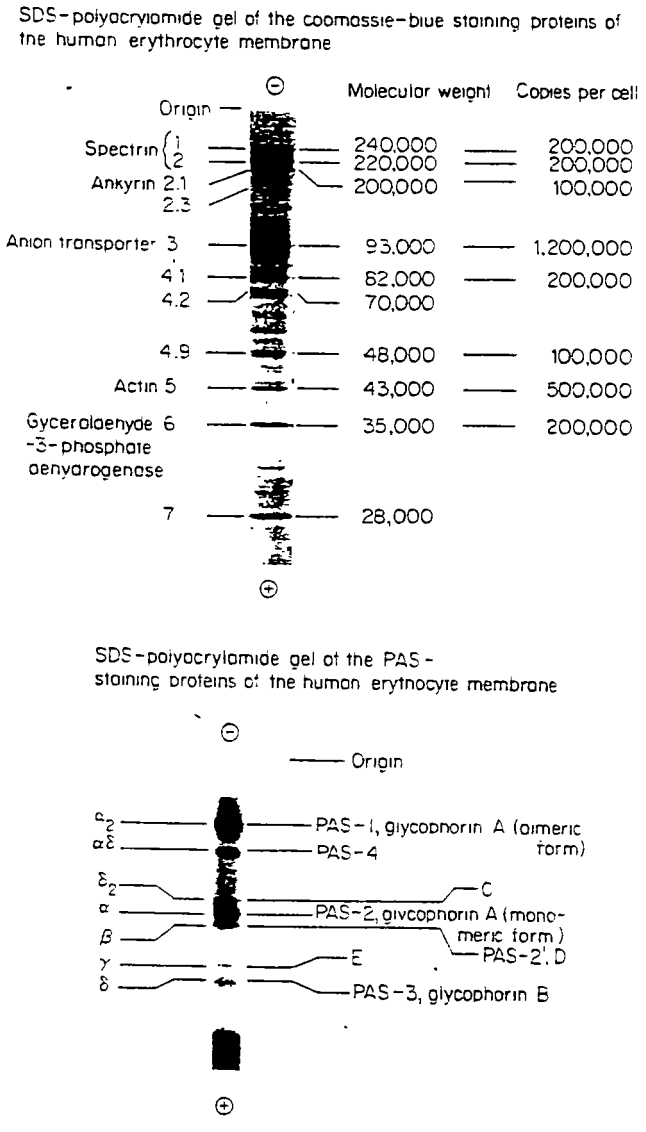


Figure 10: Proteins of the human erythrocyte membrane. Typical SDS-PAGE gels with either Coomassie blue to identify proteins (a) or with PAS reagents to identify carbohydrates residues on glycoproteins (b). The Greek letters in (b) identify each glycoprotein bands in terms of their known composition, consisting of monomers, dimers or hybrids of α, β, γ and δ polypeptide chains. (From reference 11)

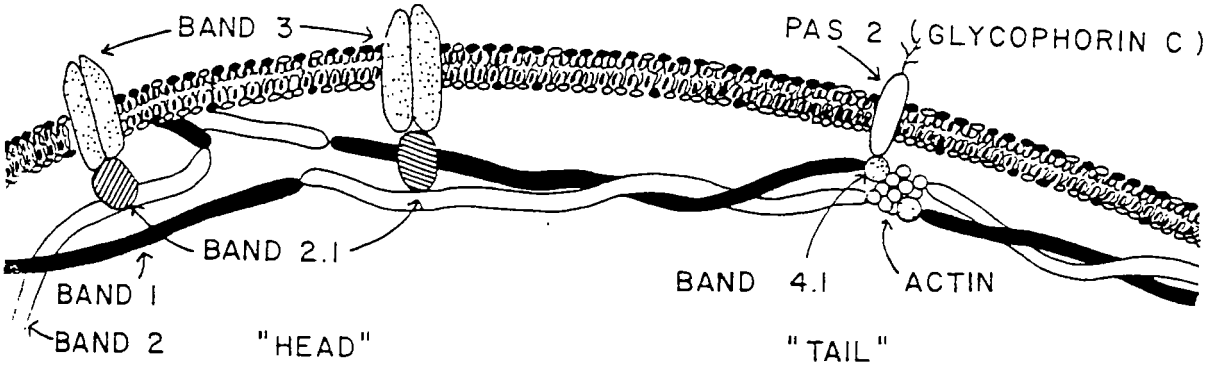


Figure 11: Diagramatic cross section of membrane bilayer and supporting "skeleton". The predominant protein of the membrane, spectrin, occurs as a heterodimer (bands 1 and 2) linked together into a fibrous network. The linkage between the "tail" ends of the dimers appears to be mediated by actin (band 5) and band 4.1. Linkage between the "head" ends of the dimers occurs by direct contact between complimentary strands of the heterodimer (the carboxy terminus of the chain and the amino terminus of the chain). Attachment of the skeleton to the membrane is produced by a specific association between band 2 of spectrin and band 3 in the lipid bilayer via the spectrin-binding protein, band 2.1 (ankyrin), near the head end of the spectrin dimer. An additional association of the skeletal complex with the lipid bilayer may be provides by a connection between spectrin and another bilayer protein, glycophorin C or A, via band 4.1. (From reference 12)

(see Figure 11): 1) side-to-side associations of Band 1 (α) and Band 2 (β) chains form heterodimers ; 2) head-to-head associations between α- chain of one dimer and the β-chain of another produce tetramers and higher oligomers ; 3) tail associations of multiple spectrin tetramers form through junctional complexes of actin and protein 4.1. The membrane skeleton is attached to the lipid bilayer through two major vertical linkages: 1) β-spectrin associates with ankyrin which in turn binds to the cytoplasmic domain of the anion transporter (Band 3) ; 2) protein 4.1 associates with actin and glycophorin.

The membrane skeleton is thought to be responsible for the shape and durability of erythrocytes for a number of reasons. First, membrane skeletons prepared from normal, sickled and elliptocytic erythrocytes retain the shape of cells from which they arise. Second, cells fragment and bud when their spectrin meshwork is disrupted. Third, when spectrin is extracted,the remaining bilayer and associated proteins disintegrate into tiny vesicles. Membrane made partially deficient in spectrin or protein 4.1 and membranes obtained from patients with deficiencies of those proteins show reductions in membrane stability and ability to withstand mechanical deformation (29).

Defects in membrane skeleton proteins have been observed in several hereditary diseases. Various types of defects have been reported in hereditary spherocytosis (HS), i.e., spectrin deficiency, abnormal spectrin-protein 4.1 binding, reduction of protein 4.2,

band 3 variant with increasing molecular weight. Hereditary elliptocytosis and hereditary pyropoikylocytosis (HPP) are associated with defect in spectrin self-association, protein 4.1 deficiency (in Caucasians), and reduction of ankyrin binding sites (29). Chassis and Mohandas (30) reported decreased stability but normal deformability of membranes from an individual with hereditary spherocytosis and defined abnormality in spectrin-protein 4.1 association. In a family with hereditary elliptocytosis and abnormality in spectrin self-association, the membranes had decreased deformability and stability. Membranes from several individuals with Malaysian ovalocytosis had decreased deformability but increased stability (30). The data showed that deformability and stability change with no fixed relationship to one another. These findings imply that different skeletal protein interactions regulate membrane deformability and stability.

1.4 **Thalassemia**

Thalassemias are the forms of inherited anemia occurring early in life and associated with splenomegaly and bone changes. The diseases constitute a subdivision of genetic disorders collectively known as hemoglobinopathies (31). In thalassemias, the synthesis of one or more of the globin chains is defective leading to an imbalance of the globin chains which causes ineffective erythropoiesis, hemolysis and a variable degree of anemia. The main varieties of thalassemias that are currently recognized are summarized in Table 2

Table 2: The main groups of thalassemias and related disorders

β Thalassemia:	β°
	Deletion
	Nondeletion
	β^{+}
	Normal Hb A_2 type 1 ("silent")
	Normal Hb A_2 type 2
δβ Thalassemia:	$(\delta\beta)^{\circ}$
	$(^{A}\gamma\delta\beta)^{\circ}$
	Hb Lepore(s)
	$^{A}\gamma\beta^{+}$ HPFH and β° thalassemia in *cis*
δ Thalassemia:	$(\delta)^{\circ}$
εγδβ Thalassemia:	$(\varepsilon\gamma\delta\beta)^{\circ}$
Hereditary persistence of fetal hemoglobin:	
	Deletion
	$(\delta\beta)$
	Nondeletion
	Linked to β-globin gene cluster
	$^{G}\gamma\beta^{+}$, $^{A}\gamma\beta^{+}$
	Unlinked to β-globin gene cluster
α Thalassemia:	α-Thalassemia 1 (α°)
	α-Thalassemia 2 (α^{+})
	Deletion
	Nondeletion

(31). Among these varieties, α- and β- thalassemias are the most common and clinically significant in Thailand.

1.4.1 α-Thalassemias

The α-thalassemias can be divided into the α-thalassemia 1 (α^0-thalassemia) in which no α–globin is produced from the α-gene complex, and the α-thalassemia 2 (α^+-thalassemia) in which the output from the complex is reduced. The α-thalassemias 1 can be subdivided further, according to the nature of the underlying molecular defect, into deletion and non-deletion types.

The α-thalassemia occurs widely throughout Africa, the Mediterranean countries, the Middle East and Southeast Asia. The α-thalassemia 1 (α^0-thalassemia) are found most commonly in the Mediterranean and Oriental populations and are extremely rare in Africa and the Middle East. However, the deletion forms of α-thalassemia 2 (α^+-thalassemia) occur with great frequency throughout West Africa, the Mediterranean, the Middle East and Southeast Asia (31). Non deletion thalassemias are less common. They have been well defined in some parts of the Mediterranean islands populations and also in parts of the Middle East and Southeast Asia.

The α-globin gene haplotype can be represented by αα, indicating the α_2 and α_1 genes,

respectively. A normal individual has the genotype $\alpha\alpha/\alpha\alpha$. A deletion involving one ($-\alpha$) or both ($--$) α genes can be further classified on the basis of its size, written as a superscript; thus $-\alpha^{3.7}$ indicates a deletion of 3.7 kb including one α-gene. In those thalassemia haplotypes when both genes are intact, that is, non-deletion lesions, the nomenclature $\alpha\alpha^{T}$ is given, but when the precise molecular defect is known, as in Hb Constant Spring, for example, $\alpha\alpha^{T}$ can be replaced by the more informative $\alpha^{cs}\alpha$.

More than 30 α-haplotypes have been described and thus potentially there are more than 465 interactions (31). Phenotypically, these result in one of four broad categories: (1) normal; (2) α-thalassemia traits in which there are mild hematological changes but no clinical abnormality; (3) HbH (β_4)disease; and (4) the Hb Barts (γ_4) hydrops fetalis syndrome. The Figure 12 summarizes the correlations of the changes in genotypes of α-thalassemias with those in the phenotypes.

1.4.1.1 <u>α-Thalassemia_1_(α^0-Thalassemia)</u>

The deletions of the globin cluster that are responsible for α-thal 1 and α-thal 2 thalassemias are illustrated in Figure 13. Deletions that cause α-thal 1 thalassemias usually remove both α_1- and α_2-genes of the α-globin gene cluster. The size of deletion ranges from 5.2 kb segment of DNA in which part of the α-globin gene is still intact to those which remove the entire

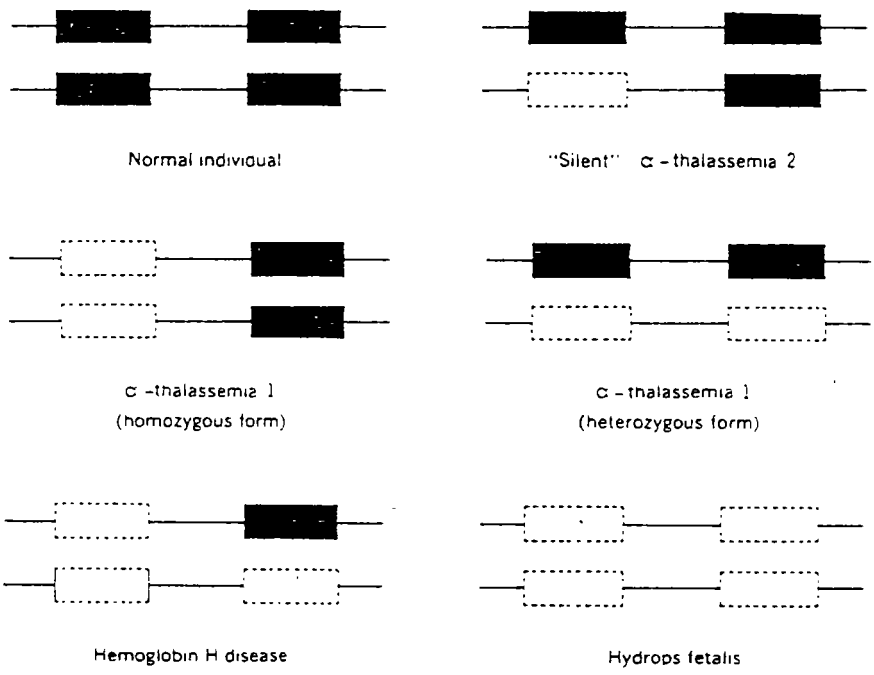


Figure 12: Genotypes of thalassemia syndrome. Gene present are represent by solid blocks, and the deleted genes by blank blocks. (From reference 67)

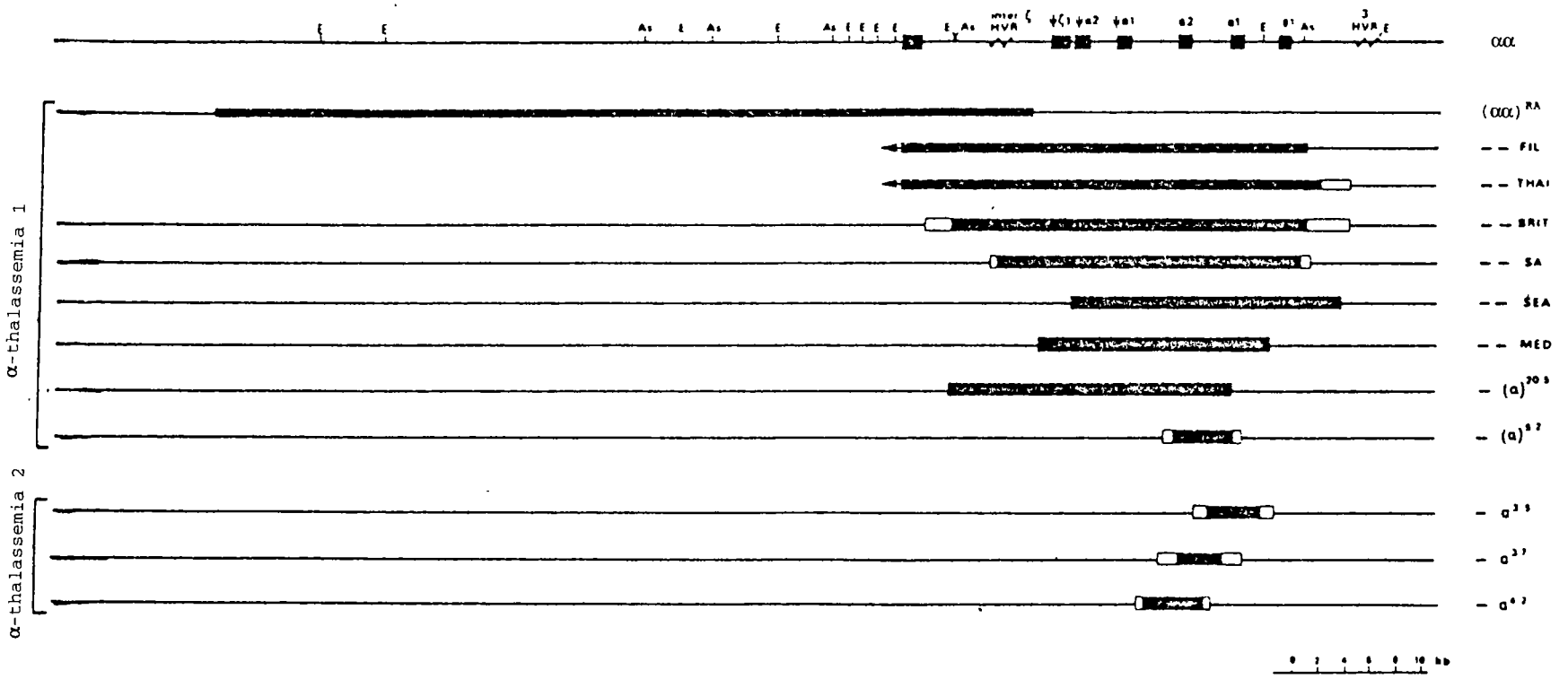


Figure 13: The deletions of the α-globin gene cluster that are responsible for α-thal 1 and α-thal 2. Some of the restriction enzyme sites and hypervariable regions that have been defined in the α-globin gene cluster are shown next to the normal α-genotype designation αα at the top of the figure. (From reference 31)

α-globin gene cluster. However, the most common α-thalassemia 1 in Thai people is found to be a deletion of about 20 kb of DNA in which both α-globin genes are removed but ζ-gene is still intact (32).

The mechanisms that cause deletions in the α-gene cluster are unknown. However, several of the 3' breakpoints fall into a 6 to 8 kb region at the 3' end of the α-globin gene complex, suggesting that this may represent a breakpoint cluster region with a high level of recombination (33). Individuals who carry one α-thal 1 thalassemia allele are asymptomatic although only two genes are functioning, but they will have certain abnormal red cell indices.

1.4.1.2 **α-Thalassemia_2_(α^+-Thalassemia)**

Deletions of the α-globin gene complex that cause α-thalassemia 2 have been detected, one involving a deletion of 4.2 kb of DNA (leftward type, $-\alpha^{4.2}$)and another removing 3.7 kb (rightward type, $-\alpha^{3.7}$). The latter has far wider distribution and is the most common form of α-thalassemia 2 in Thailand (32).

The mechanisms that cause α-thalassemia 2 have been proposed (34,35) and are shown in the Figure 14. The two α-genes are located within two highly homologous regions and each region can be divided into three non-homologous subsegments referred to as X, Y, and Z. The duplicated Z boxes are 3.7 kb apart and the X boxes 4.2

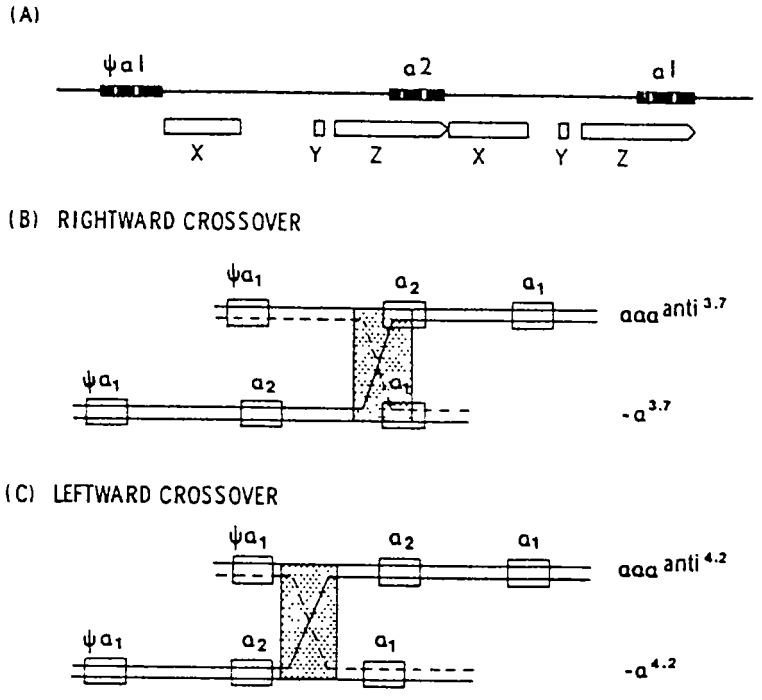


Figure 14: The mechanisms for the production of the common deletion forms of α-thalassemia 1: (A) the normal α-globin gene cluster showing the homology boxes X, Y, and Z; (B) the rightward crossover through the Z bones, give rise to the 3.7 kb deletion and a chromosome with 3 α-globin genes; (C) the leftward crossover through the Z boxes, give rise to a 4.2 kb deletion and a chromosome containing 3 α-globin genes. (From reference 31)

kb apart. Misalignment and reciprocal crossover between these segments at meiosis can give rise to chromosomes with either single (-α) or triplicated (ααα) globin genes. Such an occurrence between homologous Z boxes deletes 3.7 kb of DNA (rightward deletion $-\alpha^{3.7}$), whereas a similar crossover between the two X blocks deletes 4.2 kb of DNA (leftward deletion, $-\alpha^{4.2}$) (36). The corresponding triplicated genearrangements are referred to as $\alpha\alpha\alpha^{\text{anti-3.7}}$ and $\alpha\alpha\alpha^{\text{anti-4.2}}$ (37,38,39). More detailed analysis of these crossover events indicates that they occur more commonly in the Z box. At least three different subtypes of $-\alpha^{3.7}$ deletions have been found, depending on exactly where the crossover has taken place (40). In subtype I, the deletion extends from 863 nucleotides 5' of the duplicated genes to the 7 bp insertion/deletion divergence within the second intron. The deletions in $-\alpha^{3.7}$ subtypes II and III extend from the second intron to the divergent segment in the 3'-noncoding region of the third exon and to the small homologous segment surrounding the poly A site, respectively. The $-\alpha^{3.7}$ subtype I is the most common form deleted in many populations including Thais (40).

1.4.1.3 Non-deletion α-thalassemia

Non-deletion α-thalassemia discovered to date is mostly caused by mutations that predominantly affect the expression of the α_2-gene, presumably because the α_2-gene expression is two to three times greater than that of the α_1-gene (41, 42, 44, 44). The mutations can be classified according to the level of gene expression

that they affect (Table 3) (31). Three types of non-deletion α-thalassemia have been detected in Thailand, two of which produce a remarkable reduction in gene product (32). The most common in this group is Hb Constant Spring which arises from mutation of the termination codon in the α_2-gene resulting in an elongation of the globin from 141 amino acids to 172 residues. Hemoglobin Suan-Dok ($\alpha_2^{109\ Leu->Arg}\beta_2$) is the less common α-globin variant occurring from a missense mutation (T->G) at codon 109 in the α_2-gene. In contrast, Hb Mahidol (Q), $\alpha_2^{74\ Asp->His}\beta_2$, is associated with the leftward deletion type of α-thalassemia 2 ($-\alpha^{4.2}$). Thalassemic phenotype associated with these Hb variants results from a significant instability of these mutated globin and upon interacting with α-thalassemia 1-gene e.g. ($--/\alpha^{CS}\alpha$), ($--/-\alpha^{Q}$) they can give rise to HbH disease (32).

1.4.2 β-Thalassemias

β-Thalassemia is extremely heterogeneous at the molecular level; over 50 different mutations have been found in association with this phenotype (Table 4). They include deletions of the β-globin gene and non-deletional mutations that may affect the transcription, processing or translation of β-globin messenger RNA. Intensive studies reveal that more than 22 different mutations can be detected at various frequencies in β-thalasemia patients in Thailand (45). They include 28 A->G, codon 19 AAC->AGC, IVS-1 nucloetide 1 G->T, IVS-1

Table 3: Nondeletion mutants that cause α-thalassemia

	Affected gene	*Affected sequence*	*Nomenclature*	*Geographic distribution*
RNA processing	α2	IVS-I donor site	$\alpha^{Hph}\alpha$	Mediterranean
	$\alpha 2^{a}$	Poly-A addition	$\alpha\alpha^{TSaudi}$	Mediterranean, Middle East
RNA translation	α2	Initiator codon	$\alpha^{Nco}\alpha$	Mediterranean
	$-\alpha^{3.7}$II	Initiator codon	$-\alpha^{3.7T}$	North African, Mediterranean
	α2	In-phase terminator	$\alpha^{MS}\alpha$	Black
	α2	Terminator codon	$\alpha^{CS}\alpha$	Southeast Asia
	α2	Terminator codon	$\alpha^{IC}\alpha$	Mediterranean
	α2	Terminator codon	$\alpha^{KD}\alpha$	India
	α2	Terminator codon	$\alpha^{SR}\alpha$	Black
Posttranslational instability	α2	Exon III (125)	$\alpha^{QS}\alpha$	Southeast Asia
	(α1 or α2)	Exon III (109)	Suan Dok	Southeast Asia
	(α1 or α2)	Exon III (110)	Petah Tikvah	Southeast Asia
	$-\alpha$	Exon I	Evanston	Black
Uncharacterized	?	Unknown	$\alpha\alpha^{T}$	Several populations
	?	Unknown	$\alpha\alpha^{T}$	Greek[b]
	$-\alpha$	Unknown	$-\alpha^{T}$	Black
	?	Unknown	$\alpha\alpha^{Karditsa}$	Greek[b]

[a] This mutation has been found in both α2-like genes on an $\alpha\alpha\alpha^{anti\ 3.7}$ chromosome from a Saudi Arabian individual.
[b] Its interaction with α^{0} thalassemia to produce the Hb Barts hydrops fetalis syndrome suggests that both α-globin genes are affected.

Table 4: The molecular basis of some of the β-thalassemia

Type of mutation	*Phenotype*	*Population*
Deletion		
1. 619 bp	β°	Indian
2. 1.35 kb	High HbA_2 β°	Black
3. 10 kb	High HbA_2 β°	Dutch
4. 4.237 kb	High HbA_2 β°	Czech
Transcriptional mutants		
5.-88 C->T	β^{+}	Black, Indian
6.-87 C->G	β^{+}	Mediterranean
7.-31 A->G	β^{+}	Japanese
8.-29 A->G	β^{++}	Black, Chinese
9.-28 A->C	β^{+}	Kurdish
10.-28 A->G	β^{+}	Chinese
Processing mutants Splice junction		
11. IVS-1 5'GT->AT	β°	Mediterranean
12. IVS-1 5'GT->TT	β°	India
13. IVS-2 5'GT->AT	β°	Mediterranean, Black
14. IVS-2 3'AG->CG	β°	Black
15. IVS-2 3'AG->GG	β°	Black
16. IVS-2 3'-17 bp	β°	Kuwait
17. IVS-2 3'-25 bp	β°	Indian
Consensus sequence		
18. IVS-1 position 5 G->C	β^{+}	Indian, Chinese
19. IVS-1 position 5 G->T	β^{+}	Greek, N.European
20. IVS-1 position 5 G->A	β^{+}	Greek, Algerian
21. IVS-1 position 6 T->C	β^{++}	Mediterranean
Cryptic splice sites in exons		
22. Codon 24 T->A	β^{+}	Black
23. Codon 26 G->A	β^{++} β^{E}	S.E. Asian
24. Codon 27 G->T	β^{+}, $\beta^{Knossos}$	Mediterranean

Table 4: (Continuted)

Type of mutation	*Phenotype*	*Population*
Cryptic splice site in introns		
25. IVS-1 position 110 G->A	β^+	Mediterranean
26. IVS-1 position 116 T->G	β°	Mediterranean
27. IVS-2 position 654 C->T	β°	Chinese
28. IVS-2 position 705 T->G	β°	Mediterranean
29. IVS-2 position 745 C->G	β^+	Mediterranean
Polyadenylation signal		
30. AATAAA->AACAAA	β^+	Black
Termination codon mutants (non-sense mutants)		
31. Codon 15 C->A	β°	Indian
32. Codon 17 A->T	β°	Chinese
33. Codon 37 G->A	β°	Arab
34. Codon 39 C->T	β°	Mediterranean
35. Codon 121 G->T	β°	Polish
Frame-shift mutants		
36. Codon 6 -1	β°	Mediterranean
37. Codon 8 -2	β°	Turkish
38. Codon 8/9 +1	β°	Indian, Iranian
39. Codon 16 -1	β°	Indian
40. Codon 41/42 -4	β°	Indian, S.E.Asian
41. Codon 44 -1	β°	Kurdish
42. Codon 73/72 +1	β°	Chinese

nucleotide 5 G->C, IVS-2 nucleotide 654 C->T, G addition in codon 8/9, C deletion in codon 41, 4 base-pairs deletion in codon 41/42, A addition in codons 71/72, codon 17 AAG->TAG (stop), codon 26 CAG->TAG (stop), codon 35 TAC->TAA, and 8 base-pairs deletion in codons 123-125. Two other mutations, C->G at position -86 and G addition in codons 14/15 have also been detected in Thai patients (46). Among these mutations, the 4 base-pairs deletion in codons 41/42, and codon 17 AAG->TAG are the most prevalent (39.8 %). The minorities are ATA nucleotide -28 A->G (3.5%), IVS-1 at 5 G->C (1.8 %), and IVS II nuclotide 654 C->T (0.9 %), respectively.

1.4.3 **Pathophysiology**

The characteristic properties of thalassemic erythrocytes are the loss of cellular deformability and membrane stability. The most obvious feature of thalassemic blood is anisocytosis and poikilocytosis which leads to an overdestruction of the erythrocytes by the reticuloendothelial system. Thalassemic erythrocytes show irregular morphology with many microcytes, occasional macrocytes and variable number of target cells (47).

The pathophysiology features of the thalssemias almost all result from a primary imbalance of globin chain synthesis. The anemia of β-thalassemia has three major components:(1) most importantly, ineffective erythropoiesis; (2) short life-span of circulating mature red cells; (3) reduction in hemoglobin synthesis, resulting in hypochromic and

microcytic red cells.

Because the primary defect in β-thalassemia is in β-globin production, the synthesis of hemoglobin F ($\alpha_2\gamma_2$) and A_2 ($\alpha_2\delta_2$) should be uneffected. Fetal hemoglobin production *in utero* is normal and is only when the neonatal switch from γ to β chain production occurs that the clinical manifestations of thalassemia first appear. However, fetal hemoglobin synthesis persists beyond the neonatal period in nearly all forms of β-thalassemia. Heterozygous β-thalassemias have all elevated level of HbA_2.

The consequences of excess non α-chain production in the α-thalassemias are quite different. Because α-chains are shared by both fetal and adult hemoglobins, defective α-chain production is manifested in both fetal and adult life. In the fetus it leads to excess γ-chain production; in adult to an excess of β-chains. Excess γ-chains form γ_4 homotetramer or Hb Bart (48), and excess β-chains form β_4 homotetramers or HbH (49). Because of the ability of γ and β chains to form stable homotetramers, there is a fundamental difference in the pathophysiology of α- and β -thalassemias. In homozygous β-thalassemia, β-globin synthesis is either absent or markedly reduced. This results in the production of an excess of α-globin chains. Unpaired α-globin chains are incapable of forming a viable hemoglobin tetramer and hence bind to membranes in red cell precursors. The membrane-bound α-globin chains are

responsible for the intramedullary destruction of red cell precursors and hence for the ineffective erythropoiesis that characterizes all the thalassemias. A large proportion of the developing erythroblasts are destroyed within the marrow in severe cases. The red cells that are released are prematurely destroyed in the enlarged spleen (47).

In the α-thalassemias the erythrocytes are not destroyed in the marrow. Thus, ineffective erythropoiesis is not characteristic of α-thalassemias. Anemia developes because of damage and destruction of these cells in the spleen, and the defects in hemoglobin synthesis cause the cells to become hypochromic and microcytic. The tissue hypoxia of anemic α-thalassemias is exacerbated by both Hb Barts and HbH; their high oxygen affinities make them ineffective oxygen carriers. Severe intrauterine oxygen deprivation in homozygous α-thalassemia 1 is reflected by the grossly hydropic state of the infant (47).

1.4.4 Biochemical Alterations in Thalassemic Erythrocytes

It has been accepted that heme attachment on the membrane causes oxidative destruction to the erythrocyte in several genetically inherited anemias including thalassemias (50, 51, 52,53). The difference in severity among α- and β-thalassemias is presumably correlated with the different properties of the two types of globin chains. In α-thalassemia, most of the

excess γ- and β- chains form stable Hb Barts (γ_4)(48) and HbH (β_4) (49), respectively. In contrast, there is no evidence so far for the presence of α-chain homotetramers and recent computer simulation of quaternary structure of tetrameric α-globin indicates that such a molecule is thermodynamically unstable (54). Free α-globin chain has a higher affinity to the membrane than does the β-chain (55). In addition, auto-oxidation rate of the α-chain is about ten times greater than the β-chain (56). Following attachment to the membrane and auto-oxidation, ferric heme is released from metHb and associates with the membrane (51, 57). The presence of ferric heme in membrane can catalyse chain reactions that generate reactive oxygen-derived free radicals species such as superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl free radical ($OH^{\cdot}$), lipid peroxides or related species (58, 59). The reactions have been known as the Fenton and the Haber-Weiss reactions. Consequently, many deleterious effects of hemin on erythrocyte membrane have been observed, i.e. spectrin-Hb crosslinking; clustering of Band 3 protein, glycophorin and ankyrin; depletion of membrane sulfhydryl groups; and lipid peroxidation (50, 57, 59).

1.4.4.1 **Alterations of Membrane Lipids**

Data have accumulated suggesting that thalassemic erythrocyte membranes are subjected to increased lipid peroxidation. An increase in the production of malondialdehyde (MDA) (60, 61), a decrease in the percentage of polyunsaturated fatty acids such as

arachidonic acid and phosphatidylethanolamine (62), and a reduction of vitamin E level (63) have been reported. Perturbations in the membrane lipids in β-thalassemic RBC membrane are reflected by reduction inlipid hydrolysis with phospholipase A_2 (64), and enhance uptake of Merocyanin 540 dye (65). Lipid peroxidation also affects asymmetric distribution of phospholipids across the lipid bilayer, *in vitro* (66, 67).

The presence of phosphatidylserine (PS) on the outer surface of thal/HbE red cell could account for the elevated level of platelet factor 3-like activity in whole blood of β-thalassemia patients (68) and also in intact erythrocytes from both α- and β-thalassemias (67). In addition, the presence of cell surface PS is suggested by the observations that both α- and β-thalassemic red cells, including HbH/CS and β-thal/HbE are phagocytized to a greater extent than normal by monocytes (69). However, there is as yet no direct evidence for the appearance of PS on the surface of thalassemic erythrocytes, although there has been a report of elevated levels of surface PE (64).

1.4.4.2 **Alterations of Membrane Proteins**

The presence of thalassemic red cells with abnormal morphologies and irregular shapes indicates that there exist defects in the cell membrane cytoskeleton (70). Examination of the thalassemic red cell membrane proteins by sodium - dodecylsulfate -

polyacrylamide gel electrophoresis (SDS-PAGE) reveals the presence of excess globin chains which are also found attach to the cytoskeletal proteins following removal of lipids and intergral proteins (71). The species of membrane-bound globin chain has a profound effect on red cell properties (55). The β-thalassemic membranes are less stable and fragment readily under shear stress whereas the α-thalassemic membranes are more stable than normal (72). Entrapment of α-globin within normal erythrocyte ghosts produces loss of cellular deformability (73).

Damages in the cytoskeletal components of thalassemic erythrocyte membranes have been reported. Studies in the Middle East patients reveal that protein 4.1 is defective in β-thalassemia intermedia, whereas ankyrin appears to be affected in α-thalassemia (74). However, these observations could not be confirmed in similar studies conducted in samples from Thai patients (75). Evidence for oxidative damage to spectrin is conflicting (57, 76), but it is unlikely that spectrin would escape damage in thalassemic red cells. Recently, decreases of sulfhydryl contents in spectrin, ankyrin and protein 4.1 have been observed in β-thalassemia/HbE (67).

1.5 The Calcium Pump of The Erythrocyte Membrane

1.5.1 The Role of Calcium in Biological System

Calcium is one of the most abundant metals in living orgnisms and accounts for 2 % of the total body weight in vertebrates. Most of it is mineralized in bones and teeth as hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and only 1 % is formed in solution in the extra- and intracellular fluids. The calcium is absorbed in the proximal small intestine and this process is regulated by vitamin D_3 (1,25-dihydroxy cholecalciferol). Parathyroid hormonne, calcitonin and vitamin D are involved in the complex control of renal calcium and phosphate excretion, skeletal calcium mobilization and homeostasis of the cellular calcium level (4). The concentration of Ca^{2+} in the cytoplasm is kept low, at 10^{-7} M to 10^{-8} M, whereas the extracellular calcium concentration isaround 10^{-3} M. External stimuli such as hormones, growth factors, light, and electricity, upon activating specific cellular plasma membrane receptors, trigger the generation of the second messengers inositol (1,4,5)-triphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ then opens the specific Ca^{2+}-channel and allow Ca^{2+} to enter the cytoplasm either from outside the cell or internal organelles such as the sarcoplasmic reticulum in muscle cells or the endoplasmic reticulum in non-muscle cells (73, 74, 75). An increased cellular calcium level triggers many intracellular processes, including fertilization, cell growth, transformation, secretion, smooth muscle contraction, sensory perception, neuronal signalling, DNA replication, DNA

repair, gene transcription and cell volume recovery (79, 80, 81, 82).

The calcium signal is transduced into an intracellular response, in part by calcium-binding proteins that are thought to be involved in the regulation of many cellular activities. These proteins may be subdivided into two groups, with distinct structural features: (1) the proteins with EF-hand motif such as calmodulin, troponin-C, parvalbumin and (2) the annexin family (83). These calcium-activated proteins may influence cellular activities by affecting phosphorylation of the key enzymes in the metabolic pathways (82).

In the erythrocytes, calcium exerts significant effects such as regulation of cell shape and plasticity, control of potassium transport via the Gardos-effect and the Na^+/K^+-pump (84). *In vitro* studies in Ca^{2+} -enriched human erythrocytes showed deleterious effects including degradation of cytoskeletal proteins (85) with changes in cell morphology (86), perturbation of phospholipids asymmetry of the membrane bilayer (87, 88), loss of cellular deformability (89), and membrane vesiculations (90, 91).

1.5.2 **The Mechanism of Calcium Transport Across Erythrocyte Membrane**

The physiological calcium concentration in human erythrocytes is between 10 and 20 mol/l and most of the calcium is attached to the cell membrane (84).

Cytoplasmic calcium concentration is in the range of 10-6 to 10-7 M, about three order of magnitude smaller than that in the plasma. Any intracellular buffering of calcium makes this large, inwardly directed gradient even steeper and maintaining this calcium distribution requires powerful defence mechanisms. These are the low passive permeability of the red cell membrane for calcium and the ATP-dependent, active calcium extrusion from the cell interior (84).

Generally, eukaryotic cells possess several calcium transporting systems (Figure 15) (92). Only three of them, viz, Ca channels, a Ca^{2+} -ATPase, and a Na/Ca exchanger, are found in the plasma membranes. The Ca channels are responsible for a controlled influx of calcium ions from extracellular space. They are the voltage sensitive channels. The Na/Ca exchanger is active in excitable tissues such as heart, brain and kidney. It is a large capacity but low affinity calcium exporting system of the plasma membrane. The Ca^{2+} -ATPase has a high affinity for calcium (K_d <0.5 μM) and is believed to play the most important role in the maintenance of the 10^4 fold gradient of Ca^{2+} between cells and medium (92).

The mechanism of calcium uptake in the erythrocyte is poorly known. The process is suspected to be mediated by a carrier because the influx curve is a saturable type (93). It can be inhibited by 8 mM Sr^{2+} or 8 mM Ba^{2+} or 150 μM Co^{2+} The Ca^{2+} influx is sensitive to verapamil suggesting that the transport system in erythrocyte is similar or identical to the slow Ca^{2+}

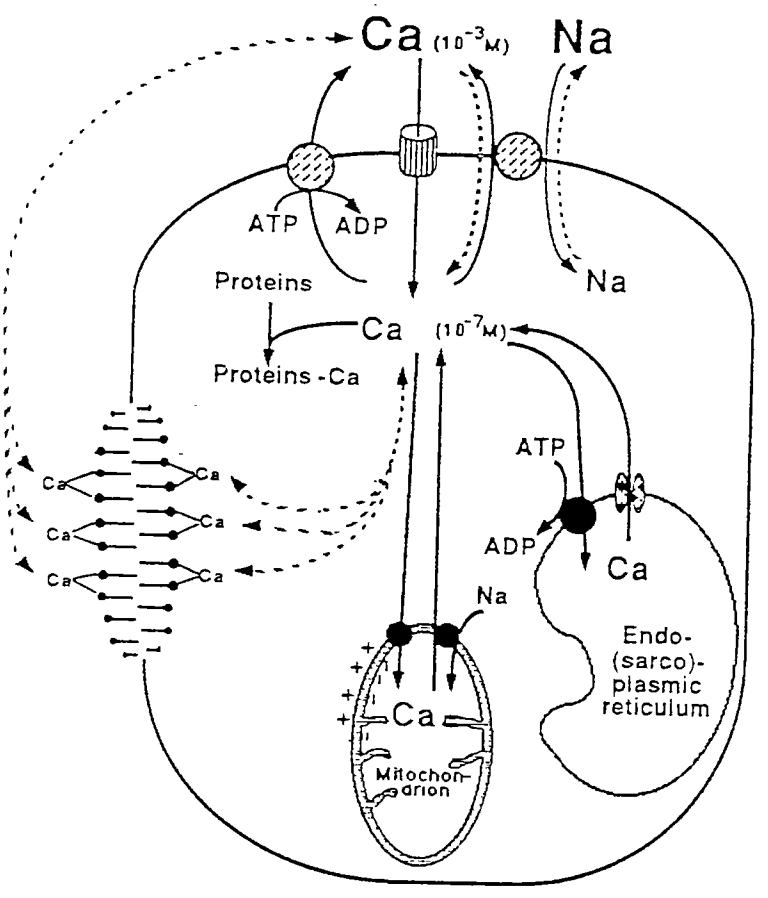


Figure 15: Calcium transporting system in eucaryotic cells. The figure shows seven transporting systems; three in the plasma membrane (the Ca-ATPase, the Na/Ca exchanger, which normally imports Ca but sometimes also exports it, and the Ca channel); two in endo (sarco) plasmic reticulum (the Ca-ATPase and the still undefined release channel); two in the the inner membrane of mitochondria (the electrophoretic uptake uniporter and the Ca releasing Na/Ca exchanger). The figure also shows the soluble Ca binding proteins of the cytosol and the acidic phospholipids of the plasma membrane as participants in the Ca buffering function. (From reference 92)

channel of excitable tissues. Moreover, the inward transport of Ca^{2+} can be inhibited by quinidine, suggesting that the gradient of K^+ ions may play a role in the system (93).

1.5.3 The Plasma Membrane Ca^{2+} -ATPase

The plasma membrane Ca^{2+} -ATPase, or Ca^{2+}/Mg^{2+} -ATPase (ATP phosphohydrolase, EC 3.6.1.3) is the only active Ca^{2+} -extrusion system in the erythrocytes (84). The enzyme belongs to the P-class of the ion-motive ATPase (94), i.e. it forms an aspartyl phosphate intermediate during the reaction cycle and is inhibited by vanadate. The pump is also inhibited by La^{3+} (K_i about 1 μM) (95). In the erythrocyte, it represents about 0.01 - 0.1 % of the total membrane proteins (96). The enzyme is a single polypeptide with a molecular mass about 138,000 and does not contain carbohydrate (97). It has high affinity for Ca^{2+} (K_m <0.5 μM) and ATP (K_m 1-2.5 μM)(95) and is stimulated by Na^+ or K^+(93).

The plasma membrane Ca^{2+} -ATPase is stimulated by direct interaction with calmodulin (CaM) (K_d about 1 nM). Other activating factors have been suggested, e.g. polyunsaturated fatty acid, acidic phospholipids, kinase-mediated phosphorylation (PKA, PKC), limited proteolysis and oligomerization (95, 98, 99, 100).

The stoichiometry between transported Ca^{2+} and hydrolysed ATP approaches 1.0 in plasma membrane Ca^{2+} -ATPase, in contrast to 2.0 in the Ca^{2+}- pump of sarcoplasmic reticulum (101, 102). The proposed

mechanism for the reaction cycle is shown in Figure 16. The enzyme in the Ca^{2+}-high affinity conformer (10 μM free Ca^{2+} = E_1 state), after binding to MgATP and Ca^{2+}, is phosphorylated at a specific aspartyl residue (residue number 475) (103). Then, the phosphoenzyme intermediate of E_1 transforms to the Ca^{2+}-low affinity conformer (E_2 -state) from which the bound Ca^{2+} ion is released. The phosphoenzyme intermediate of E_2 conformer is then dephosphorylated; the mechanism may be associated with the incoming ATP molecule which also accelerates E_2 to E_1 transition. It has been proposed that vanadate (VO_4^{3-}) inhibition involves the step of E_2-> E_1 transition by interacting with the E_2 conformer and thus blocks the last step of thereaction cycle (104). Lantanum ion (La^{3+})inhibits the Ca^{2+} -pump of the red cell by arresting the proteins in a phosphorylated form, that is, La^{3+} blocks the transition between E_1-P and E_2-P (105).

Recent cloning experiments (106, 107, 108, 109) have shown that there are at least five isoforms and over ten variants of the Ca^{2+}-pump (Table 5). The Ca^{2+}-pump isoforms are encoded by a multigene family and additional variability are produced by alternative RNA splicing of each gene transcript (95). Among these varieties, the isoform PMCA4 represents the major Ca^{2+} -pump in mature human erythrocytes. Other isoforms found in mature human erythrocytes are PMCA1b and PMCA2 (95, 98). The gene for PMCA1 has now been located on human chromosome 12 (q21 - q23) and that for PMCA4 on human chromosome 1 (q25 - q32) (95, 98, 110). The gene for human isoform PMCA2 is probably located on

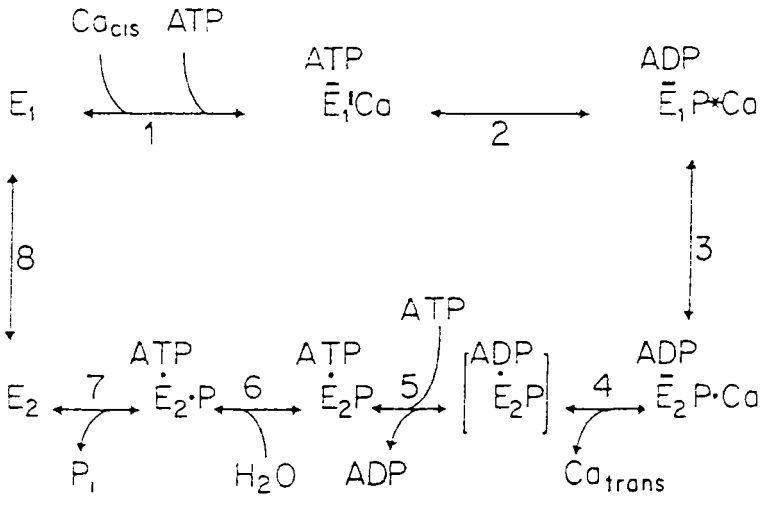


Figure 16: Proposed reaction cycle of the Ca pump in plasma membrane and sarcoplasmic reticulum. E_1, E_2: conformations of pump protein; translocation occurrence step 3; EP, covalent bond: (.) low and (-) high affinity non-covalent ATP, ADP of Ca^{2+} bond; (*) "occluded" Ca^{2+} ; [ ], "ADP-insensitive" E. In forward mode (clockwise): invading arrows, preceding E has increased affinity; emerging arrows, preceding E has decreased affinity. Mg^{2+} is not shown: it probably enters at step 1 and is liberated at step 7; step 3 (4) is decisive for acceleration by Mg^{2+} . It is unclear what happens between ATP.E_2 and ATP/E_1 (step 8). Step 6 (7, 8?) is decisive for acceleration by ATP at low affinity site. (From reference 101)

Table 5: The plasma membrane Ca^{2+} pump isoforms. The information presented is deduced from cDNA isolated from either rat or human cDNA libraries.

Isoform	*Species*	*Splicing variants*	*Number of residues*	*Calculated mass*
PMCA 1	rat, human	1a	1176	129,500
		1b	1120	134,700
		1c	1249	137,800
		1d	1258	138,800
PMCA 2	rat, human	2f	1198	132,600
		2g	1243	136,800
		2h	1212	134,000
PMCA 3	rat	3a	1159	127,300
PMCA 4	human	4a	1170	129,400
		4b	1205	133,900
		4g	1169	130,300
(PMCA 5)	bovine	?	71	7,700

chromosome 3 (95).

The general topography of the Ca^{2+} -pump in the plasma membrane has been elucidated by prediction from the hydrophathy plots (111), epitopes specific monoclonal antibodies labelling (112), and chemical labelling techniques (95, 113) and is shown in Figure 17. Ten putative transmembrane domains have been identified, concentrated in the N- and C- terminal portions of the pump. Most of the pump mass protrudes into the cytoplasm, with very short loops connecting the putative transmembrane (TM) domains on the external side. Three main units protrude into the cytosol, between TM domains 2 and 3, 4 and 5, and from domain 10. The first corresponds to the "transducing unit" which permits the couple of ATP hydrolysis to the transport of Ca^{2+}. The second large protruding domain contains the aspartyl phosphate site (Asp -475 in the human isoform PMCA 1b) and the site of ATP binding (Lys -609 in the same isoform). The C-terminal of this domain contains a "hinge" region, formed by two α-helices around the axis of which a motion occurs to bring the phosphorylation domain next to the bound ATP. This unit also contains a receptor site for the "autoinhibitory" calmodulin-binding domain. The C-terminal protruding domain contains the calmodulin-binding sequence flanked by two α-helical acidic stretches which have been hypothesized to "channel" Ca^{2+} to its catalytic site. It also contains the site of phosphorylation by PKA and PKC. The PKA site (Ser -1178) is present only in one of the two isoforms expressed in human erythrocytes.

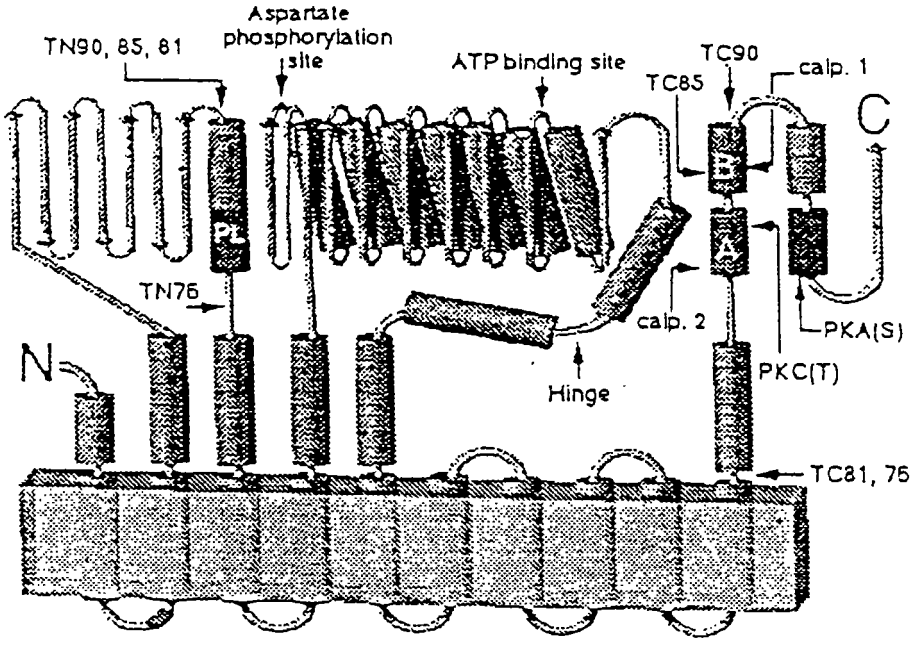


Figure 17: Architecture of the Ca pump. Cylinders indicate predicted α-helices, arrows β-sheets. The two calmodulin binding sub-domains are indicated by A and B. Calp 1 and calp 2 are the two sequential sites of calpain cleavage. PKA (S) and PKC (T) are sites of serine and threonine kinase phosphorylation. TC is the notation for the C-terminal clevage by trypsin, TN that of the N-terminal cleavage. PL is the domain responsive to acidic phospholipids. (From reference 95)

1.5.4 Calcium Homeostasis in Thalassemic Erythrocyte

Accumulation of calcium in thalassemic erythrocytes has been reported. The magnitude of increased cellular calcium as determined by atomic absorption spectrophotometry is 3-8 fold in non-splenectomy, and 7-20 fold in splenectomy (114,115, 116). However, the cause of calcium accumulation in thalassemic erythrocytes is obscure. Wiley (117) reported increased Ca^{2+}-permeability in erythrocytes from β-thalassemia major. Other investigators (114,116) reported normal Ca^{2+}-permeability in β-thalassemia intermedia from the Mediterranean, despite that ^{45}Ca influx rate was increased in RBCs from splenectomized patients and two different rates were observed between light and dense cell fractions.In addition, by calculation, they also reported normal cytosolic free Ca^{2+} ion concentration in these erythrocytes. In another hand, Shalev et al (115) reported normal Ca^{2+}/Mg^{2+}-ATPase activity in both splenectomized and non-splenectomized β-thalassemia intermedia patients. From these observations, the investigators (115, 116) suggested that the Ca^{2+}-pump was not impaired in β-thalassemia and that most of the increased cellular calcium ions were compartmentalized within the endocytic inside-out vesicles.

1.6 Objectives of the Thesis

An increase in total cellular calcium content has been observed in several types of hereditary hemolytic disorders including favic glucose-6-phosphate dehydrogenase deficiency (118, 119), sickle cell disease (120, 121), and hemoglobin C disease (114). Increased Hb auto-oxidation in association with deposition of hemichromes, free hemin and non-heme iron have been observed in these genetically hemolytic diseases (122, 123). Membrane damages by oxidative effects of bound hemin have been described by a generation of reactive oxygen free-radicals (50). Oxidative inactivation of the erythrocyte calmodulin-stimulated Ca^{2+}/ Mg^{2+} -ATPase by hemin (124), non-heme iron (125), and oxygen free-radicals generating oxidants (126, 127) have been studied in normal human erythrocytes. Increased susceptibility of the plasma membrane Ca^{2+}/Mg^{2+} -ATPase to increased oxygen free-radicals has been observed in sickle (128, 129) and G-6-PD deficient (130) erythrocytes. Progressive inactivation of Ca^{2+}-pump has also been observed during the sickling process (131). Loss of calmodulin stimulation (132, 133) and reduction in the activity (134) have also been reported in Ca^{2+}/Mg^{2+} -ATPase of the sickle erythrocyte membranes. These evidences suggest that oxidative damages to the plasma membrane Ca^{2+}-pump are involved in the accumulation of calcium ions in the sickle erythrocytes.

Evidences of erythrocyte membrane injury due to oxidative effects of membrane-bound hemichromes, hemin, and non-heme iron have been described in both

human (57) and mouse (135) thalassemias. However, an involvement of the Ca^{2+}-pump inactivation to an accumulation of cellular calcium in thalassemic erythrocytes is conflicting (115, 116). It is unlikely that the Ca^{2+}/Mg^{2+}-ATPase in thalassemic erythrocyte membranes is able to escape from oxidative damages.

In this study, the properties of the Ca^{2+}/Mg^{2+}-ATPase in thalassemic erythrocyte membrane were investigated extensively. The enzyme activity was examined independently to the presence and absence of Ca^{2+} ions and calmodulin activator. The Arrhenius properties (the responses of enzyme activity to changes in the temperature) and the Ca^{2+}-dependency of the Ca^{2+}-pump were also studied. The data from thalassemic Ca^{2+}-pump were compared with those obtained from the pump in normal erythrocyte membranes that were pretreated with oxygen free-radicals generating oxidants.

CHAPTER 2

MATERIALS AND METHODS

1. Subjects

Normal control subjects were healthy persons working in the Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand. All of them possessed normal hematologic indices and hemoglobin typing. Patients with thalassemia of all types were identified and selected at the out-patient department of Siriraj Hospital, Mahidol University, Bangkok, Thailand. None of them had recieved blood transfusion for at least 3 months prior to blood donation.

2. Chemicals

All chemicals used in this study were of analytical graded and were listed alphabetically as followings.

Name of Chemicals	*Companies*
Acids	
Acetic acid, glacial	Merck
Hydrochloric acid, concentrated	Merck
Perchloric acid, 60 %	Carlo-Erba
Phosphoric acid, 85 %	Carlo-Erba
Sulfuric acid, concentrated	Merck
Trichloroacetic acid	Riedel-de-Haen

Name of Chemicals	*Companies*
Buffers	
HEPES (N-[2-HYdroxyethyl]piperazine-N'-[2-ethansulfonic acid])	Sigma
Tris (tris[Hydroxymethyl]aminomethane	Sigma
MOPS (3-[N-morpholino]propanesulfonic acid	Sigma
Chelating Agents	
EDTA (Ethylenediaminetetraacetic acid	Sigma
EGTA (Ethyleneglycol-bis[-aminoethylether]-N,N,N',N'-tetraacetic acid	Sigma
HEDTA (N-Hydroxyethylethylenediamine-triacetic acid)	Sigma
Dyes	
Bromphenol blue	Metheson
Coomassie brilliant blue R-250	Sigma
Malachite green	Sigma
Serva blue G 250	Serva
Research	
Electrophoresis materials	
Acrylamide	Sigma
Ammonium persulfate	Sigma
Bis arylamide	Sigma
TEMED (N,N,N',N'-tetramethyl ethylene-diamine	Sigma
Sodium dodecylsulfate (SDS)	Sigma

Name of Chemicals	*Companies*
Enzymes and Proteins	
Bovine serum albumin (BSA)	Sigma
Calmodulin (CaM)	Sigma
Concanavalin A (Con A)	Calbiochem
Lactate dehydrogenase (LDH)	Sigma
Pyruvate kinase (PK)	Sigma
Trypsin	Sigma
Soybean trypsin inhibitor	Sigma
Xanthine oxidase	Sigma
Nucleotides and High-Energy Compounds	
ATP, disodium salt	Fluka
Inosine	Sigma
β-NADH	Sigma
Phosphoenolpyruvate (PEP)	Sigma
Organic Solvents	
Dibutylphthalate oil, $\rho = 1.042\text{-}1.045$	Sigma
Ethanol, 95 %	Carlo-Erba
Chloroform	Carlo-Erba
Glycerol	Carlo-Erba
Isopropanol	Mallinckrodt
Oxidants	
Diamide	Sigma
Hypoxanthine	Sigma
p-Chloromercuribenzoate	Sigma
Phenazine methosulfate	Sigma
Phenylhydrazine hydrochloride	Sigma

Name of Chemicals	*Companies*
Sodium periodate	Sigma
tert-Butylhydroperoxide	Sigma
Radioactive Materials	
$^{45}CaCl_2$	NEN
[1-^{3}H]-L-Glucose	NEN
γ-^{32}P-ATP	NEN
Scintillation Materials	
POPOP (2,2'-p-Phenylene-bis-[5-phenyloxazole])	Sigma
PPO (2,5-Diphenyloxazole)	Sigma
Toluene	Merck
Triton X-100	Sigma
Miscellaneous	
Ammonium molybdate	Sigma
$CaCl_2 .2H_2O$	J.T. Baker
α-Cellulose	Sigma
Citric acid	Sigma
Citrate, trisodium	Carlo-Erba
Dithiothreitol(DTT)	Sigma
D-Glucose	BDH
Iodoacetamide	Sigma
KCl	Merck
KH_2PO_4	Riedel-de-Haen
$LaCl_3$	Fluka
$MgCl_2 .6H_2O$	Riedel-de-Haen

Name of Chemicals	*Companies*
$MgSO_4 .7H_2O$	Mallinckrodt
$MnCl_2 .4H_2O$	Sigma
$NaBH_4$	Sigma
NaCl	Carlo-Erba
$NaHCO_3$	Sigma
Na_2HPO_4	Merck
NaOH	Merck
Ouabain	Sigma
Percoll	Sigma
Sucrose	Fischer
Urea	Fluka

3. **Instruments**

Instrument	*Companies*
Liquid Scintillation Counter, LS-1801	Beckman
Microcentrifuge, bench-topped	Eppendorf
Mini Protein II Slab Gel	Bio-Rad
pH meter	Corning
Spectronic-21	Milton-Roy
Spectrophotometer UV-160 A	Shimadzu
Superspeed Refrigurated Centrifuge, RC-5	Sorvall
Water bath, 37 °C	Haake
X-rays Photographic film, Kodak-X-OMAT	Kodak

4. Collection of Blood Samples

Blood samples to be studied should be fresh and intact as much as possible. Acid citrate dextrose solution was used as anticoagulant.

Reagent:

acid citrate dextrose:

trisodium citrate, dihydrate	2.79	g
citric acid, monohydrate	1.01	g
D-glucose	2.5	g
distilled water, adjusted to	100	ml

Procedure

Blood sample was withdrawn by venipuncture using disposible plastic syringe. About 20 ml volume of whole blood was collected in a disposible plastic capped-tube containing 2 ml of the acid citrate dextrose solution. The tube was kept on ice until used. Generally, blood sample was studied within the day collected. However, normal blood sample which was collected in a blood transfusion bag and kept at 4 °C for 3 weeks could be used without significant alteration in the activity of Ca^{2+} -ATPase.

5. Fractionation of Thalassemic Erythrocytes

Usually,bloodsample from a splenectomized patient constitutes of a heterogeneous population of red blood cells. To study the difference in the magnitude of alterations in the properties of the Ca^{2+} -ATPase among diferent populations of the red blood cells, whole blood sample from a splenectomized patient was fractionated by centrifugation in "Percoll" density gradient (136, 137, 138).

Reagents:

Stock solution A;
5.263 g % BSA in H_2O
Stock solution B;
5.263 g % BSA in "Percoll"
Stock Buffer C;
2.66 M NaCl
0.09 M KCl
0.20 M HEPES, pH 7.4
Stock Buffer D;
2.66 M NaCl
0.09 M KCl
0.20 M HEPES, pH 8.5
Isotonic solution A;
38 ml stock solution A
2 ml stock buffer C
Isotonic solution B;
38 ml stock solution B
2 ml stock buffer D
(final 95 % Percoll, pH 7.4)

Washing buffer;

133 mM NaCl

4.5 mM KCl

10 mM HEPES, pH 7.4

50 % Percoll, 5 ml;

2.65 ml isotonic solution B

2.35 ml isotonic solution A

70 % Percoll, 5 ml;

3.70 ml isotonic solution B

1.30 ml isotonic solution A

Procedure

In a plastic round-bottom centrifuge tube, 5 ml volume of 70 % Percoll was placed to the bottom and 50 % Percoll was carefully placed on top.

Whole blood sample from a splenectomized patient was adjusted to 30 % PCV by centrifugation and removal of some of the plasma.

A volume of 1.0 ml of the blood sample was carefully layered on top of Percoll gradient.

The tube was centrifuged at 1,100 xg, 5 minutes, at room temperature, using a swinging rotor.

The top-most layer, which contained plasma and white blood cells, was carefully removed and discarded by aspiration.

The 50% Percoll layer containing abnormal and young red blood cells was collected and was labelled as "light cell fraction" ($\rho < 1.090$).

The 70 % Percoll layer containing more mature red blood cells was collected separately and was labelled as "dense cell fraction" (ρ> 1.090).

Each cell fraction was washed 3 times to removed the Percoll with the washing buffer (HBS; HEPES-Buffered Saline).

6. Oxidative Treatement of Normal Red Blood Cells

In order to mimic the oxidative stress condition in thalassemic erythrocytes and to study the effects of oxidising agents on the properties of the Ca^{2+}-ATPase, intact normal human erythrocytes were treated with different types of oxidants. The oxidants used in this study were: diamide; a cross-linking agent, p-chloromercuribenzoate (PCMB); a sulfhydryl agent, phenazine methosulfate (PMS) and phenylhydrazine(PHZ); producing superoxide radicals (O_2^-) and tert-butylhydroperoxide (TBH) and xanthine oxidase (XO); generating peroxide radicals ($\cdot HO_2^-$).

Reagents:

Stock solution 250 mM diamide;
0.0431 g diamide/ml HBSS
Stock solution 25 mM PCMB;
0.0090 g PCMB/ml HBSS

Stock solution 200 mM PMS;

0.0613 g PMS/ml HBSS

Stock solution 200 mM PHZ;

0.0149 g PHZ/ml HBSS

Stock solution 100 mM TBH;

12.9 l 70 % TBH/ml HBSS

Stock solution 30 mM Hypoxanthine;

0.0410 g Hypoxanthine

0.5 ml 2 M KOH

9.5 ml HBSS

Xanthine oxidase 40 U/ml

Hank's Balanced Salt Solution (HBSS) 1.0 L ;

glucose	1	g		
NaCL	8	g	136.9	mM
KCl	0.4026	g	5.4	mM
Na_2HPO_4	0.0468	g	0.3	mM
KH_2PO_4	0.0408	g	0.3	mM
$MgSO_4 .7H_2O$	0.0986	g	0.4	mM
$CaCl_2$	0.1911	g	1.3	mM
$MgCl_2$	0.1017	g	0.5	mM
$NaHCO_3$	0.3528	g	4.2	mM

pH 7.4

Washing Buffer;

130	mM KCl	9.7	g/L
10	mM Tris-HCl	1.21	g/L

pH 7.4

Procedure:

Normal whole blood sample was spun in a Sorvall RC-5 centrifuge machine,using SS-34 rotor, at 5,000 rpm, 4 °C, for 10 minutes. Plasma and buffy coat were discarded by aspiration. Packed red blood cells were washed 3 times with washing buffer to clean up plasma and white blood cells. Washed red blood cells were resuspended to 20 % V/V in HBSS. An aliquot of 15 ml of the RBC suspension was added with the oxidant stock solution to obtain a given final concentration.

The suspension was incubated with gently agitation at 37 °C for 1 hour, in a water bath.

The oxidised red blood cells were washed 3 times to removed the oxidant by the same centrifugation process.

To study the effects of oxidants on erythrocyte membranes in cell-free system, aliquot of 5 ml membrane preparation (see Procedure 7) containing 1 mg protein/ml was added with the oxidant stock solution to obtain a given final concentration.

The suspension was incubated in a water bath at 37 °C, for 15 - 30 minutes.

The oxidised membranes were washed 3 times with Buffer 3 (see Procedure 7). Then, the membranes were resuspended in 1.0 ml Buffer 3.

7. Preparation of Calmodulin-Deficient Erythrocyte Membranes

In order to observe the basal ATPase activity and to study the effects of added calmodulin on activation of the erythrocyte membrane Ca^{2+} -ATPase, the calmodulin-deficient erythrocyte membranes were required. Preparation of the calmodulin-deficient membranes was followed the method described by Niggli, et al (139).

Regents:

Buffer 1;	130	mM KCl
	10	mM Tris-HCl, pH 7.4
Buffer 2;	1	mM EDTA
	10	mM Tris-HCl, pH 7.4
Buffer 3;	130	mM NaCl
	0.5	mM $MgCl_2$
	0.05	mM $CaCl_2$
	10	mM HEPES, pH 7.4

Procedure:

The whole procedure was carried out at 4 °C and the specimen was kept in an ice bath.

Whole blood sample was spun in a RC-5 Sorvall centrifuge machine at 5,000 rpm, 10 minutes, 4 °C, using a SS-34 rotor. Plasma and buffy coat were discarded by aspiration. Packed red blood cells were resuspended in 5

volumes of Buffer 1, and the cells were washed 3 times to clean up the plasma and white blood cells.

One volume of washed erythrocytes were added to 10 volumes of the lysis Buffer 2. The mixture was shaken vigoriously for 1 minute prior to centrifugation at 24,000 x g for 30 minutes. The supernatant and the dense creamy brown "button" at the bottom of the centrifuge tube were discarded by aspiration.

The ghost membranes were washed 2 times by the same procedure in Buffer 2.

The clean ghosts were washed once in Buffer 3.

The calmodulin-deficient membranes were resuspended in Buffer 3 to the final protein concentration of about 4 mg/ml. The membrane suspension could be stored -80 °C up to 1 month without significant difference in the enzyme activity.

8. **Protein Assay**

For the purpose of rapid estimation of protein concentration in the membrane preparation, and to avoid the interference of HEPES buffer in the membrane preparation to the Lowry's method, the dye-binding method using Coomassie brilliant blue G-250 as described by Read and Northcote (140) was used.

Reagents:

Ethanol, 95 %	94	ml
Serva Blue G (SERVA research grade 35050)	0.1	g
H_3PO_4 ,85 %	200	ml

Stock solution bovine serum albumin (BSA)
10.0 mg/ml

Procedure:

Preparation of the Bradford's Reagent.

Serva Blue G-250 (0.1 g) was stirred in 94 ml of the 95 % ethanol for 30 minutes or until the dye was completely dissolved.

H_3PO_4, 200 ml, was added with stirring and followed by distilled water to the final volume of 2 liters.

The solution was filtered through Whatman no.1 filter paper.

The reagent was kept in a dark brown bottle at room temperature.

Setting up a Standard Curve.

The stock standard solution BSA (10mg/ml) was diluted to give final concentrations of 0.1 to 1.0 mg/ml.

Aliquot of 50 l protein sample was mixed with 2.5 ml Bradford's reagent, in a glass test-tube. The tube was let stand 10 minutes at room temperature. The absorbance at 596 nm was read within 10 minutes by Spectronic 21 spectrophotometer.

Duplicated measurements were performed and the standard curve was drawn from the average values.

The protein standard curve was shown in Figure 18.

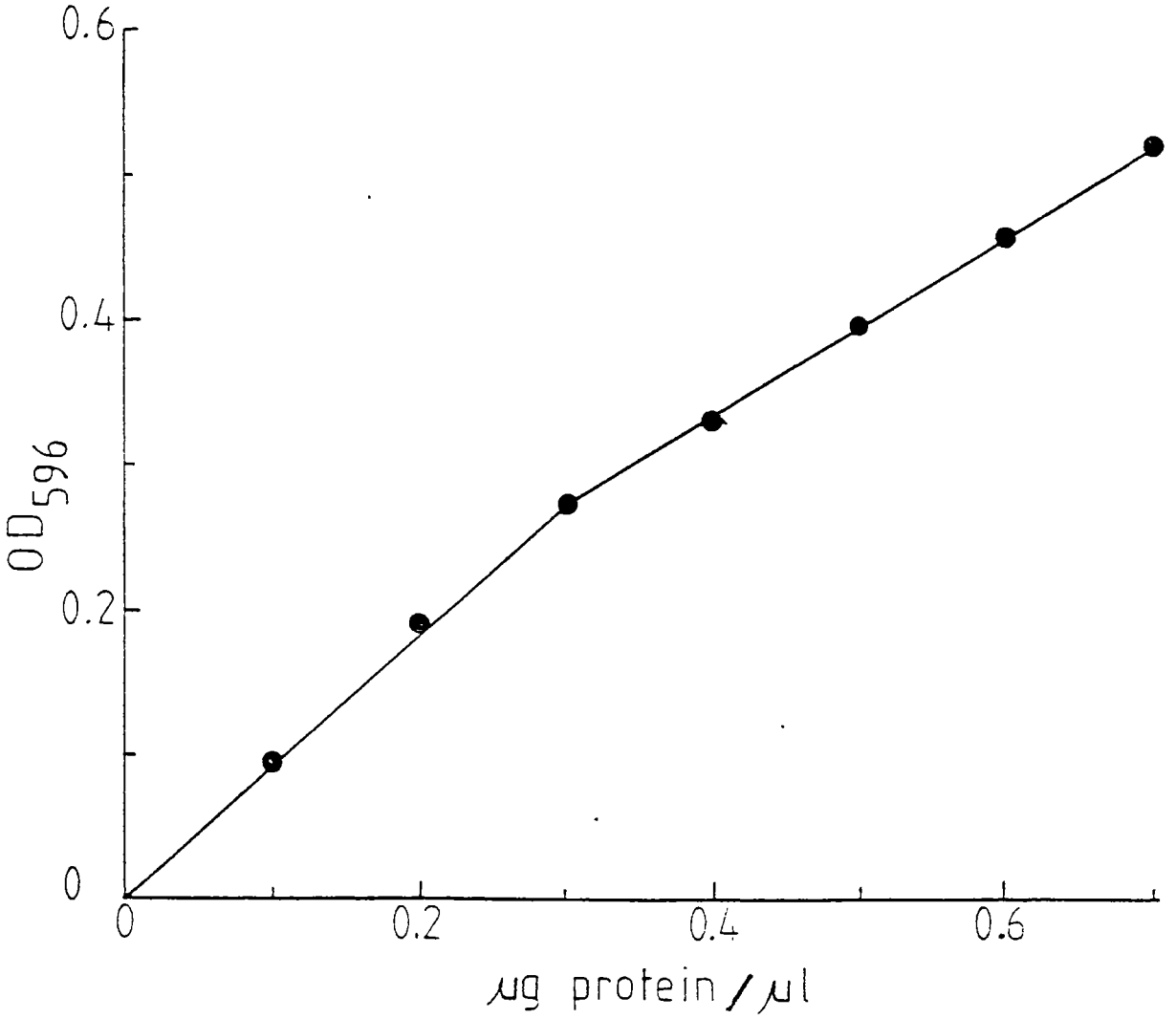


Figure 18: The protein standard curve. The protein was assayed by the Coomassie brilliant blue dye-binding as described in "Materials and Methods". Bovine serum albumin (BSA) was used as the standard protein.

Determination of Protein in Membrane Preparation.

The erythrocyte membrane preparation was diluted 1:50 prior to protein determination.

The volume proportions of the diluted membrane preparation to Bradford's reagent was the same as working with the standard BSA solution.

9. **Assay for the Membrane Phospholipids**

For the determination of phospholipids, the membrane preparation was extracted by isopropanol/ chloroform (11:7) (141). The phospholipids extract was digested in concentrated H_2SO_4, and oxidised to form inorganic phosphate by $HClO_4$ (142). The inorganic phosphate was then formed green complex with molybdate and malachite green (143).

Reagents:

Isopropanol
Chloroform
5 M H_2SO_4
60 % $HClO_4$
Malachite green hydrochloride
4.2 % Ammonium molybdate in 4 N HCl
34 % Sodium citrate
10 % Triton X-100
10.0 mM KH_2PO_4 standard solution

Procedure:

Preparation of Lanzetta's Reagent.

A. MG solution, 0.045 %:

An amount of 0.45 g malachite green hydrochloride was dissolved in 1 L of distilled water.

B. AM solution, 4.2 % ammonium molybdate in 4 N HCl.

C. Working color reagent:

The MG solution was mixed with the AM solution at a volume ratio of 3:1 with continuous stirring by a magnetic stirrer for 20 minutes.

The solution was filtered through Whatman no. 5 filter paper.

A volume of 0.08 ml of 10 % Triton X-100 was added for each 100 ml of the color reagent.

The Lanzetta's reagent was prepared freshly before
used.

Setting of the Phosphate Standard Curve.

The stock standard solution 10.0 mM KH_2PO_4 was diluted 1:100 to the concentration of 1 nmole/10 µl. This solution was then prepared to 1:2 serial dilutions.

Aliquots of 0.1 ml of the 1:2 serial dilutions were mixed with 1.6 ml aliquots of the Lanzetta's reagent, in clean glass test-tubes. The mixtures were

let stand for 1 minute. A volume of 0.2 ml 34 % citrate solution was added into each tube with well mixing.

The reaction was let stand for 30 minutes at room temperature.

The absorbance was read at 630 nm against water blank in Shimadzu UV 160 A spectrophotometer, using quartz cuvettes.

The measurements were done in duplication and the standard curve was drawn from the average values.

The phosphate standard curve was shown in Figure 19.

Determination of Phospholipids in Erythrocyte Membrane Preparations.

A volume of 10 µl erythrocyte membrane preparation was mixed with 90 µl distilled water in an 1.5 ml Eppendorf tube. Isopropanol, 550 µl, was added and the tube was let stand with occasional shaking for 1 hour.

Chloroform, 350 µl, was then added and the tube was let stand for next 1 hour, with occasional shaking.

The tube was centrifuged at top speed in a microfuge machine for 5 minutes.

Two volumes of 200 µl of the organic extract were pipetted into 15 ml Pyrex test-tubes. The organic solvent was evaporated to dryness at 80 °C, in a hot-air incubator.

Aliquotsof 20 µl 5 M H_2SO_4 and 60 µl 60 % $HClO_4$ were added to the lipid residual. The tubes were heated

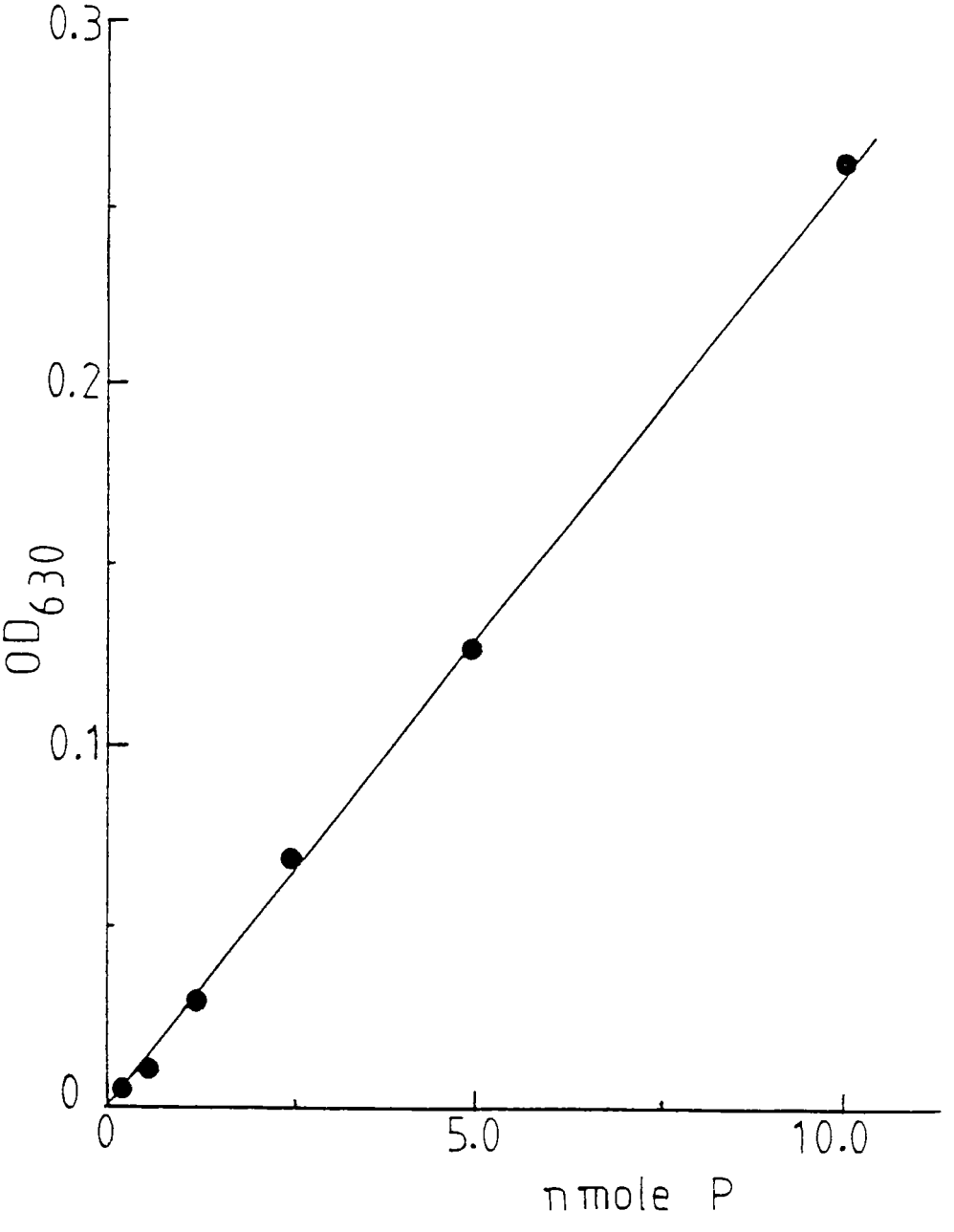


Figure 19: The phosphorus standard curve for the determination of membrane phospholipids as described in "Materials and Methods". KH_2PO_4 was used as the standard phosphate.

over a direct flame, in a fume cabinet, for a few minutes until the solution was colorless and all the oxidant residual was decomposed.

After cooling, 0.1 ml volume of distilled water was added into the tube, following by 1.6 ml of Lanzetta's reagent and 0.2 ml of 34 % sodium citrate solution, as described in the setting up of the phosphate standard curve.

Calculation of the Phospholipids Concentration.

From OD_{630} reading, the amount of phosphate in the reaction could be read from the standard curve. Let X nmole P was the standard curve reading. Then, the concentration of phospholipids in the membrane preparation, P, was 0.45 X moles/ml.

10. Controlled Proteolysis of Erythrocyte Membranes

To study the effects of the calmodulin-binding domain on the Arrhenius properties of the Ca2+ -ATPase, the erythrocyte membrane preparation was treated with trypsin under controlled conditions to removed the calmodulin - binding domain (144). The Arrhenius properties of the truncated enzyme was studied in comparison to the normal control.

Reagents:

Proteolysis medium;

Composition	*Vol. in 100 ml total*	*Final Concentration*
3 M KCl	2.5 ml	75 mM
1 M HEPES	3.0 ml	30 mM
50 mM EDTA	0.2 ml	100 M
Adjusted to pH 7.0		

Trypsin solution, 10 μg/μl;

1 mg trypsin

0.1 ml proteolysis medium

Soybean trypsin inhibitor solution, 10 μg/μl;

0.015 g soybean trypsin inhibitor

1.5 ml proteolysis medium

Procedure:

The erythrocyte membrane preparation (see Procedure 7) was washed once with the proteolysis medium by centrifugation (see Procedure 7). The membrane pellets were resuspended in the proteolysis medium to the concentration of 1 mg protein/ml.

An aliquot of 5 ml of the membrane suspension (5 mg total membrane proteins) was incubated with 10 μl of trypsin solution in a water bath, 37 °C, for 0-4 minutes.

A volume of 100 l of soybean trypsin inhibitor solution was added to stop the proteolysis. The tube was kept on ice.

The membranes were pelleted by centrifugation and washed 3 times the Buffer 3 (see Procedure 7) containing soybean trypsin inhibitor (10 μg/μl).

The trypsin-treated membranes were resuspended in 5 ml of Buffer 3 and stored at -80 °C.

11. Assay of the Ca^{2+}/Mg^{2+} -ATPase Activity

The Ca^{2+} /Mg^{2+} -ATPase activity was assayed by the coupling enzyme method. The assay involves the coupling of the ATPase reaction with the reaction of pyruvate kinase (PK) with phosphoenolpyruvate (PEP), and lactate dehydrogenase (LDH) with β-NADH. The reaction sequence is as follow:

	PEP		Pyruvate		Lactate
ATPase		PK		LDH	
ATP	ADP + P	ATP		β-NADH	NAD^+

Since PK and LDH are in excess, the rate limiting step in the coupled enzyme assay is the ATPase-catalysed conversion of ATP into ADP + P_i. The oxidation of β-NADH is measured at 340 nm. This is directly related to the ATPase activity. For each mole of β-NADH oxidised, one mole of ATP is hydrolysed.

The composition of the coupled enzyme medium was followed the procedure described by Sarkadi, et al (144).

Reagents:

Bovine brain calmodulin solution;

1 μg/5 μl membrane storage buffer

Coupled enzyme medium;

Stock solution	*Final volume in 100 ml*		*Final concentration*	
50 mM HEDTA	1.0	ml	0.5	mM
50 mM EGTA	1.0	ml	0.5	mM
10 mM Ouabain	1.0	ml	0.1	mM
50 mM $CaCl_2$	1.2	ml	0.6	mM
3 M KCl	4.0	ml	120	mM
1 M HEPES	3.0	ml	30	mM
100 mM $MgCl_2$	2.5	ml	2.5	mM
ATP	0.0870	g	1.5	mM
PEP	0.0293	g	1.25	mM

The pH was adjusted to 7.4 with 2 M KOH.

β-NADH	0.0140	g	0.2	mM
PK (5,000 U)	56	μl	1	U/ml
LDH (10,000 U)	11	μl	1	U/ml

This medium contained 10 μM free Ca^{2+} ions.

Procedure:

Measurement of the Enzyme Reaction.

A volume of 1.0 ml of the coupled enzyme medium was pipetted into a quartz cuvette. The cuvette was placed in the temperature-controlled chamber of the Shimadzu UV 160A spectrophotometer and was incubated at a given temperature for 5 minutes. Aliquot of 10 µl of the erythrocyte membrane preparation (40 µg membrane proteins) was added into the medium and mixed well. The rate of β-NADH oxidation was monitored by following the declining slope of the absorbance at 340 nm against the coupled enzyme medium placed in the reference chamber of the spectrophotometer. The kinetics programme was used in the analysis.

Calculation of the Enzyme Specific Activity.

Calculation of the Ca^{2+}/Mg^{2+}-ATPase specific activity was based on the Beer-Lambert's law.

Taking the equation;

$$A = \varepsilon Cl$$

where, A; the rate of the decrease in OD_{340}, dA/min, l; the light path length, 1.0 cm, and ; ε the extinction coefficient of β-NADH, 5.26 $mole^{-1}.L.cm^{-1}$.

Then, the rate of oxidation of β-NADH by 10 µl of the membrane preparation could be written as:

$$C = \frac{dA}{5.26} \quad \text{mole/L/min}$$

Given, P represented the concentration of phospholipids in the membrane preparation in μmoles/ml. The specific activity of the Ca^{2+}/Mg^{2+}-ATPase could be calculated from the equation:

$$C = \frac{-dA}{5.26 \cdot P} \times 10^5 \quad \mu\text{moles ATP/ mole membrane PL/min.}$$

Studies of the Activation Effect of Calmodulin.

For the study of the activation effects of calmodulin on the Ca^{2+}/Mg^{2+}-ATPase activity, 10 μl aliquots of membrane suspension were preincubated with 5 μl of the calmodulin solution, at 37 °C for 3 minutes, before addition to the coupled enzyme medium.

Studies of the Effects of Ca^{2+} ions Concentration on the Ca^{2+}/Mg^{2+}-ATPase Activity.

To study the dependency of the Ca^{2+}/Mg^{2+}-ATPase activity on changes in Ca^{2+} ions concentration, the given volumes of the stock solution $CaCl_2$ were added into the coupled enzyme medium to the given final concentrations of $CaCl_2$.

The Ca^{2+}/Mg^{2+}-ATPase activity was assayed as described above.

12. Arrhenius Studies and Calculation of Activation Energy

The Arrhenius study shows the relationships of the enzyme activity to the change in temperature (145). Increase of temperature almost invariably increases the rate of a chemical reaction to a marked extent; for homogeneous processes the specific rate is usually increased by a factor of about two or three for every 10 °C rise of temperature. The most satisfactory method for expressing the influence of temperature on reaction velocity can be derived by plotting the logarithm of the specific rate, i.e., log *K*, against the reciprocal of absolute temperature, i.e., against 1/T. Then, the variation of the rate constant of a reaction with temperature can be expressed by means of the **Arrhenius equation:**

$$\log K = \log A - \frac{E}{2.303\ R\ T},$$

whereas A is sometimes called the **frequency factor**, and E is the **energy of activation** of the reaction.

Procedure:

The Arrhenius Study.

The enzyme activity was assayed as described in Procedure 11. The temperature in the cuvette chamber

of the Shimadzu UV 160 A spectrophotometer was controlled by the Pelteir system. The enzyme activity was assayed at every 2 degree Celcius, ranging from 14 to 40 °C.

The logarithms of the specific activity were plotted against the reciprocals of absolute temperature. The plots that showed correlation coefficient greater than 0.980 were assumed to be a straight line (146).

Calculation of Activation Energy of the Reaction.

To calculate the activation energy, it requires the plot of log activity against 1/T to be linear, the slope being equal to -E/2.303 R. The constant E, which is characteristic of the reaction and determines the influence of temperature on the reaction rate, can thus be calculated from the value of this slope.

13. **Study of the Phosphoenzyme Intermediate**

Formation of phosphoenzyme intermediate indicates normal activity of the plasma membrane Ca^{2+}/Mg^{2+}-ATPase. The phosphoenzyme intermediate can be demonstrated using [γ-^{32}P]ATP in the presence of Ca^{2+} and La^{3+} following by analysis in specific, acidic SDS-PAGE and autoradiography (147,148,149).

Reagents:

Reaction medium (3x concentrated) :

300 mM KCl

60 mM MOPS, pH 6.9

Plasma membranes suspension in 50 mM MOPS, pH 6.9, 5 mg protein/ml

1 mM $CaCl_2$

1 mM $LaCl_3$

20 mM EGTA-K

8 % TCA

100 µM ATP

Labelled ATP : 10 µl 100 µM ATP

2.5 µl [γ-^{32}P] ATP (10 mCi/ml)

20.5 µl distilled water

Reaction medium (2x) :

Test 1 : 600 µl 3x medium

90 µl EGTA

210 µl water

Test 2 : 600 µl 3x medium

90 µl $CaCl_2$

210 µl water

Test 3 : 600 µl 3x medium

90 µl $CaCl_2$

90 µl $LaCl_3$

120 µl water

30 % acrylamide, 0.8 % bis-acrylamide

10 % ammonium persulfate (APS)

TEMED

Running gel buffer :

0.5 M Tris, adjust to pH 6.8 with H_3PO_4

0.8 % SDS

Stacking gel buffer :

0.5 M Tris, adjust to pH 5.8 with H_3PO_4

1.2 % SDS

Acidic sample buffer :

0.5 g SDS, 0.6 g DTT, 4 g Urea, 1 ml Stacking buffer, pH 5.8, diluted to 10 ml, added a few mg of bromphenol blue

Electrode buffer, pH 6.8 :

20 g HEPES (0.168 M)

1.8 g Tris (0.03 M)

0.5 g SDS (0.1 %)

500 ml distilled water

Staining solution :

1.25 g Coomassie blue R-250

500 ml methanol

100 ml acetic acid

400 ml water

Destaining solution :

700 ml methanol

200 ml Acetic acid

Water added to 2 L

5 % v/v glycerol in destaining solution

Procedure

To an 1.5 ml Eppendorf tube was added 25 µl medium 2x, x µl plasma membranes suspension (containing 50 µg membrane protein), and (20 - x) µl water. The tube

was placed at room temperature (25 °C) for a few minutes. The reaction was started by addition of 5 µl labelled ATP (final concentration was 30 µM). The tube was stirred using a Vortex mixer for 10 seconds and then incubated for another 10 seconds. The reaction was stopped by adding 0.6 ml of cold 8% TCA. The tube was placed on ice. The precipitate was settled down by centrifugation for 15 minutes in a cold room. Supernatant was discarded and the precipitate was washed once with 0.8 ml of ice-cold distilled water without resuspending of the pellet. The precipitate was then dissolved in 25 µl of the acidic sample buffer.

The phosphoenzyme was analysed on 0.75 mm thick, 6% Sarkadi acidic SDS-acrylamide gel, using a Mini Gel Protein II system of Bio-Rad. The gel composition was as following:

Composition	*Running*	*Stacking*
30 % acrylamide, 0.8 % Bis	1.0 ml	0.25 ml
running gel buffer	0.63 ml	--
stacking gel buffer	--	0.16 ml
dist. H_2O	3.37 ml	1.59 ml
TEMED	5 µl	5 µl
10 % APS	25 µl	25 µl

The gel was stained for 15 minutes and destained overnight. After soaking the gel in glycerol-containing destain solution for 5 minutes, the gel was let dry at room temperature. Autoradigraphy was done by exposure

the dry gel to an X-rays film for 5 to 7 days, at -70°C, using an intensifying screen.

15. Effect of Oxidation on Membrane Permeability of Human Erythrocytes

Accumulation of calcium in the oxidative thalassemic erythrocytes may also be a consequence from changes in membrane permeability. To study this effect, normal human erythrocytes were oxidised by phenazine methosulfate (PMS) in ATP depleted condition and the changes in membrane permeability were investigated by using ^{45}Ca ions and [1-^{3}H]-L-glucose. This experiment was accomplished by following the method described by Lew and brown (150) and Lew and Garcia-Sancho (151).

Reagents

Buffer 1 : KCl buffer, pH 7.4

140 mM KCl
10 mM NaCl
10 mM Tris - HCl, pH 7.4

Buffer 2 : NaCl buffer, pH 7.4

145 mM NaCl
5 mM KCl
10 mM Tris - HCl, pH 7.4

Buffer 3 : Washing saline solution

150 mM NaCl
1 mM EGTA, pH 7.4

Iodoacetamide

Inosine

Stock solution 200 mM PMS

Stock solution $^{45}CaCl_2$, 1 μCi/μl

Stock solution [1-^{3}H]-L-Glucose, 1 μCi/μl

Dibutylphthalate oil, density 1.042 - 1.045

5 % TCA

Scintillation fluid:			
	PPO	24	g
	POPOP	0.3	g
	Toluene	2	L
	Triton X-100	2	L

Procedure

Normal human whole blood was washed 3 times with Buffer 1 by centrifugation in Sorvall RC-5 high speed centrifuge machine using SS-34 rotor, at 5,800 x g for 10 minutes. Plasma and buffy coat were removed.

Washed packed red cells were resuspended to 20 % v/v suspension in Buffer 1, containing 10 mM inosine and 1 mM iodoacetamide. Aliquots of 10 ml of the red cells suspension were incubated at 37 °C, for 30 minutes.

After addition of phenazine methosulfate to a final concentration of 2-4 mM, the cells were incubated further for 1 hour. Then, the cells were washed twice with Buffer 1 to remove excess oxidant.

The oxidised red cells were resuspended to 10 % v/v in either Buffer 1 or Buffer 2. Aliquots of 10 ml of the 10 % v/v suspension were then added with 10 μCi of ^{45}Ca ions and 10 μCi of [1-^{3}H]-L-Glucose. The cells

were incubated at 37 °C in a water bath with gently shaking.

At a given time intervals, 500 µl aliquots of the suspension were pipetted into Eppendorf tubes containing 400 µl dibutylphthalate oil. The tubes were centrifuged at room temperature for 15 seconds. The buffer and oil were removed using Pasteur pipette. The cells were washed 3 times with 500 µl washing saline solution and 400 µl dibutylphthalate oil. The side wall of the tube was cleaned during each washing with cotton swab. Then, 500 µl of 5 % TCA solution was added and the precipitate was settled down by centrifugation. The clear supernatant, 400 µl, was mixed with 4.6 ml of the scintillation fluid in a glass vial and the radioactivity was recorded in a Beckman liquid scintillation counter.

CHAPTER 3

RESULTS

1. The Optimal Concentrations of the Coupled Enzyme_Medium Composition

The optimal concentrations of the composition in the coupled enzyme assay medium were determined as described by Niggli et al (139). In this medium, the calculated concentration of free calcium ions was about 10 μM. The total volume of the medium in each enzyme assay was 1.0 ml and the amount of protein in added membrane preparation was 10 μg. The proportion of calmodulin to membrane protein in normal membrane preparation that gave rise to maximal activation was determined. As shown in Figure 20, the proportion was 1:40. The concentration of ATP that gave maximal velocity was 1.5 mM (Figure 21). Assaying of the enzyme activity under these conditions, the velocity of the enzymatic reaction was constant for more than 30 minutes.

Because the membrane preparations from thalassemic erythrocyte samples were usually contaminated with variable amount of hemoglobin, the specific activity of the enzyme was expressed as μmole ATP hydrolysed/μmole membrane phospholipid/minute.

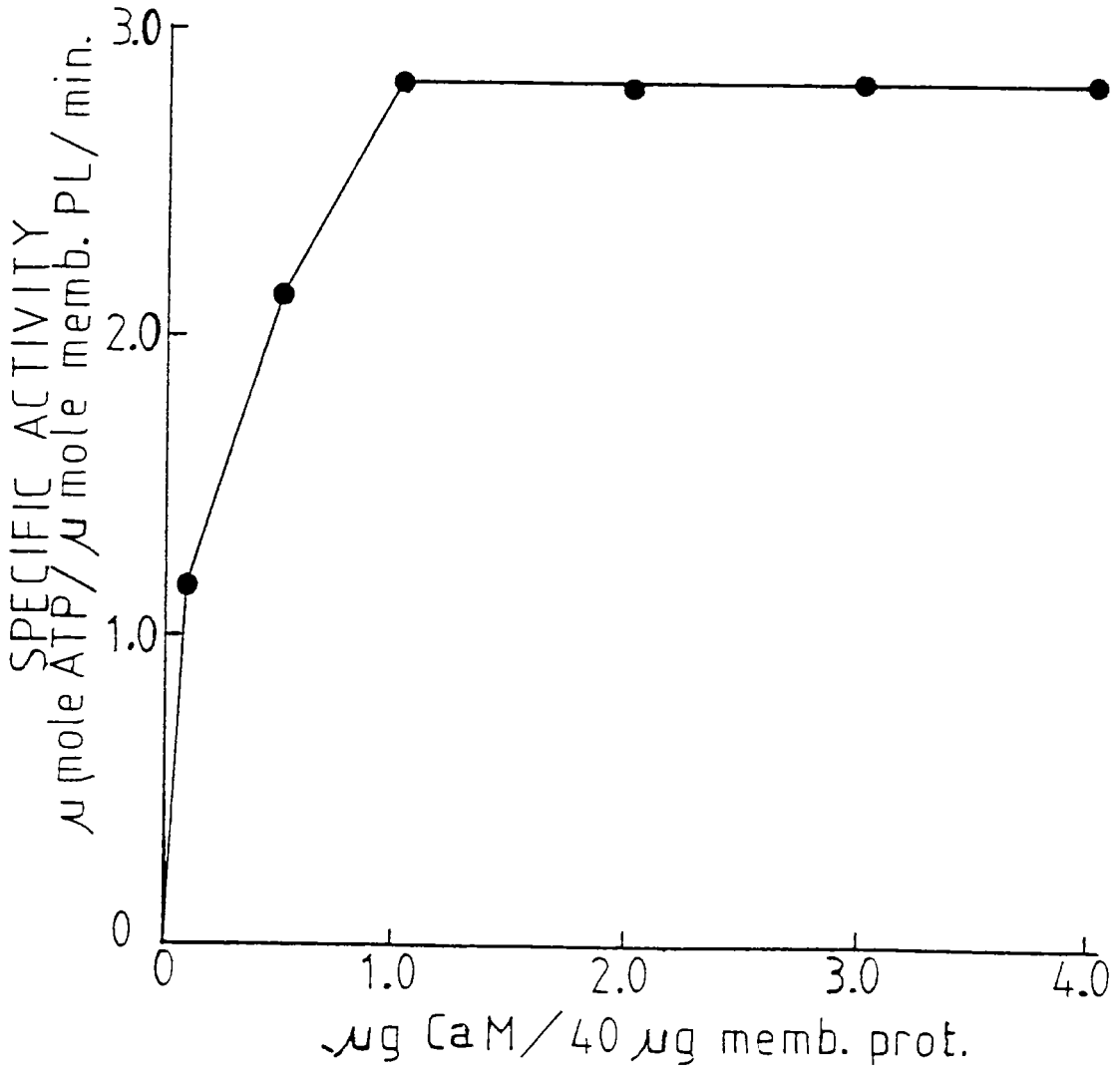


Figure 20: Effect of calmodulin on red blood cell membrane Ca^{2+} -ATPase. Membrane suspension (40 μg protein/ 10 μl) was preincubated with calmodulin for 3 minutes, 37 °C. The Ca^{2+} -ATPase activity was assayed by coupled enzyme method as described in "Materials and Methods". The ATPase activity in absence of calmodulin was substracted from those in presence of calmodulin.

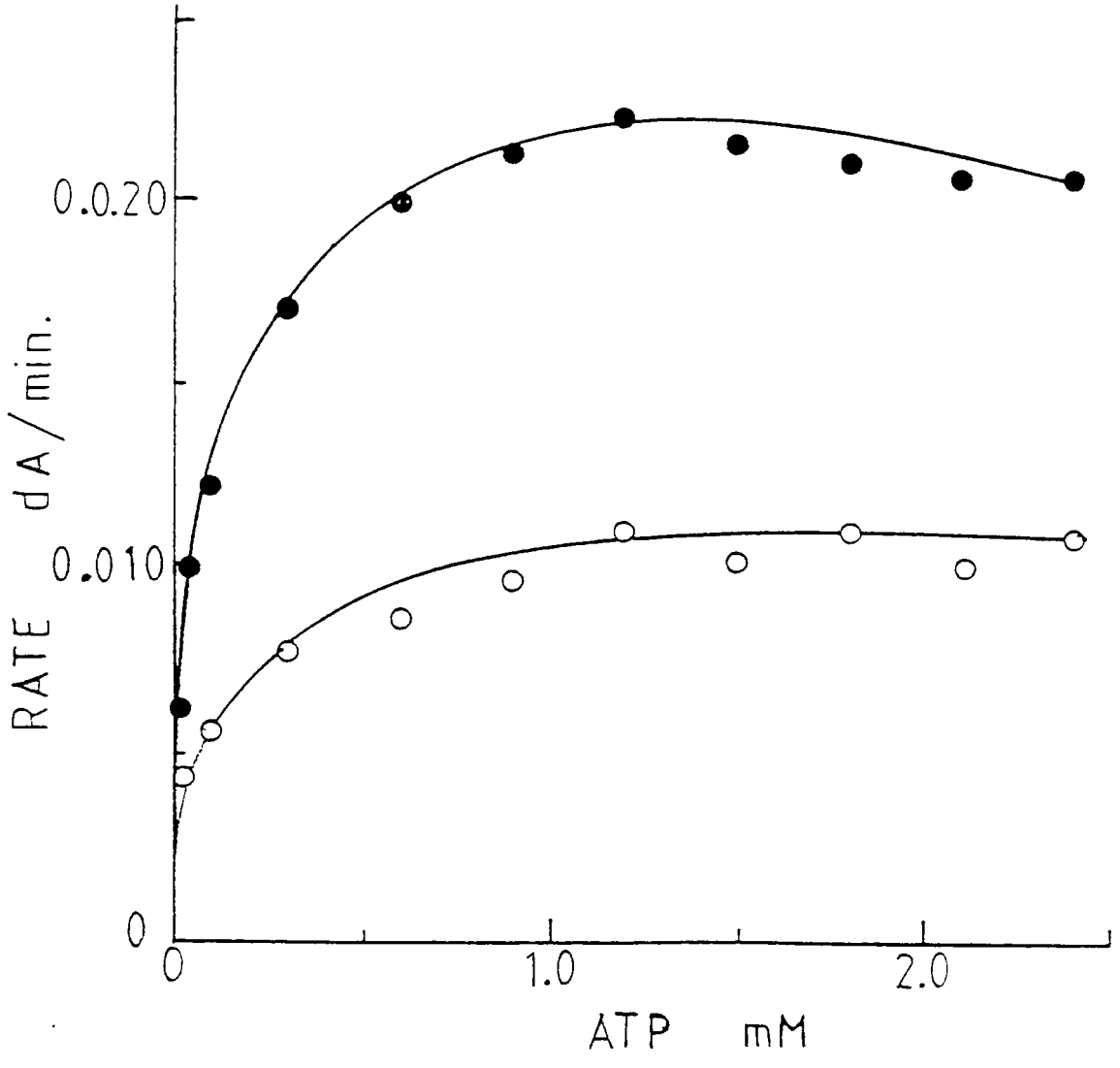


Figure 21: Effect of ATP on red blood cell membrane Ca^{2+}-ATPase. Aliquots of 1 ml of the coupled enzyme medium containing variable concentrations of ATP were preincubated at 37 °C, 3 min. The reaction was started by addition of membrane suspension (10 µl containing 40 µg protein) with, -●-, or without, -o-, addition of calmodulin (1 µg).

2. The Arrhenius Profiles of Ca^{2+}/Mg^{2+}-ATPase Activity from Normal Erythrocyte Membranes

The calmodulin-deficient ghosts were prepared from normal erythrocytes as described under "Materials and Methods". The membrane preparations were assayed for the Ca^{2+}/Mg^{2+}-ATPase activity by PK/LDH coupled enzyme method, both with and without addition of bovine brain calmodulin at every 2 degree Celcius between 14-40 °C. The logarithmic values of specific activity (μmole ATP hydrolysed/μmole membrane phospholipid/minute) were plotted against the reciprocal of absolute temperature. The results shown in the Figure 22 represent a typical normal Arrhenius profile of the enzyme activity in response to changes in the temperature. Four different normal blood samples were studied. In the absence of calmodulin, the Arrhenius profile was linear. The calculated activation energy, E_{act}, for the ATPase reaction was 16 kcal (Table 6). Under activation by bovine brain calmodulin, ATPase activity was increased more than three fold and the profile showed an Arrhenius break at the temperature 32 °C. The calculated activation energy above the break was similar to that without calmodulin activation, whereas the E_{act} value below 32 °C was 26 kcal, i.e. about 1.6 fold greater.

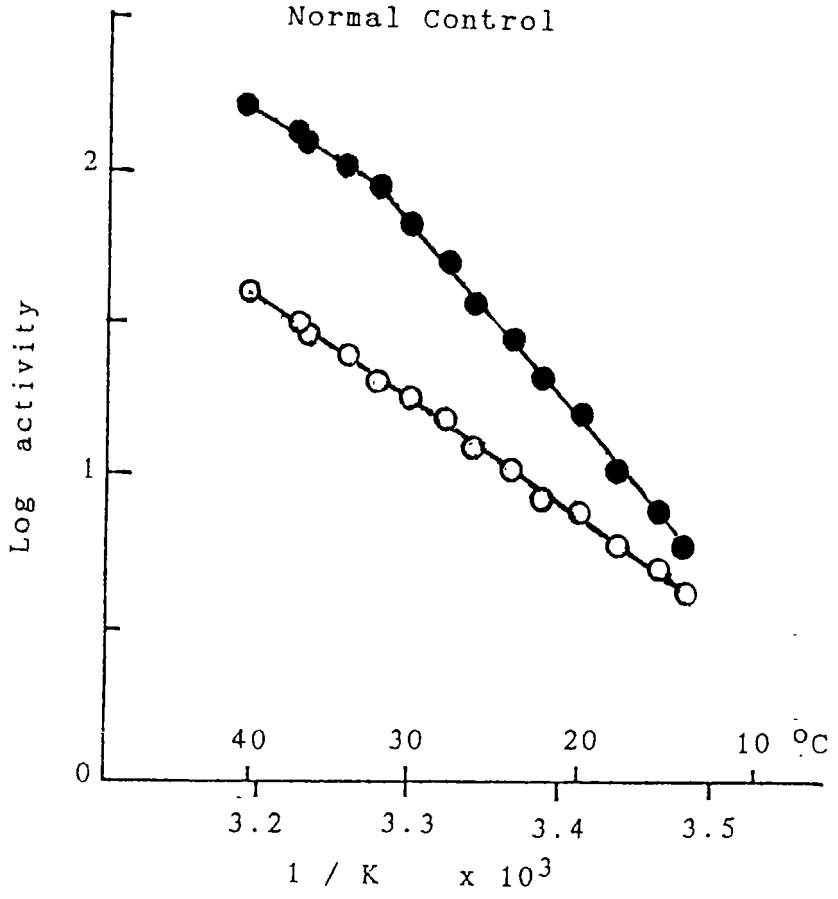


Figure 22: The Arrhenius profiles of normal erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase. Membranes were prepared as described in "Materials and Methods". The enzyme activities were assayed by PK/LDH coupled enzyme method. The specific activity was μ mole ATP/ μ mole phospholipids/minute; -●- and -o- , with and without addition of calmodulin, respectively.

Table 6: Observed Arrhenius breaks and calculated energy of activation, E_{act}, of the Ca^{2+} /Mg^{2+} - ATPase activity in normal and thalassemic erythrocyte membranes.

Ca^{2+}/Mg^{2+} ATPase	E_{act} (Kcal)			
	Without CaM	With CaM		
		Arrhenius Break,°C	E_{act} (below the break)	E_{act} (above the break)
Normal (n=4)	15.84±1.35	31.9±0.1	25.92±0.95	15.57±0.71
Trypsinized (n=2)		32.5±0.5	24.19±0.02	13.12±0.99
B/E;1/3		31.1	21.56	10.11
2/3		—	15.88±0.23	—
B/E,Spx (n=4)		32.8±0.6	17.09±1.68	9.32±1.93
Homo B (n=2)		—	18.72±1.05	—
HbH (n=2)		32.5±0.5	21.28±0.78	6.25±1.03
HbH/Hb CS (n=1)		—	16.57	—

3. The Arrhenius Profiles of Ca^{2+}/Mg^{2+}-ATPase from Thalassemic Erythrocytes

ATPase from membrane preparations from 4 cases of splenectomized β-thalassemia/HbE, 3 cases of nonsplenectomized β-thalassemia/HbE, 2 cases of homozygous β-thalassemia, 2 cases of HbH disease (α-$thal_1/\alpha$-$thal_2$), 1 case of 4 base-pairs deletion type β-thalassemia/HbE, and 1 case of HbH/HbCS were studied. The Arrhenius profiles of Ca^{2+}/Mg^{2+}-ATPase activity are shown in Figure 23-28. In every case, the enzyme did not respond to calmodulin stimulation. Indeed, the enzyme activity was already activated. The specific activities of the enzyme were extraordinarily high in splenectomized β-thalassemia/Hb E samples. The Arrhenius break at 32 °C could still be observed in cases of HbH and β-thal/Hb E, but disappeared in other types of thalassemia. However, the values of energy of activation, E_{act}, below the Arrhenius break were reduced and became closer to the value above the break of normal membrane preparations (Table 6). Also, in those thalassemia samples in which the Arrhenius break disappeared, the E_{act} values became similar to that of normal samples in the absence of calmodulin activation (Table 6). The alterations in the Arrhenius profiles suggested that in every case of thalassemias the calmodulin-binding domain of the erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase was absent. In addition, the disappearance of the Arrhenius break at 32 °C and the reduction in the energy of activation also suggested a

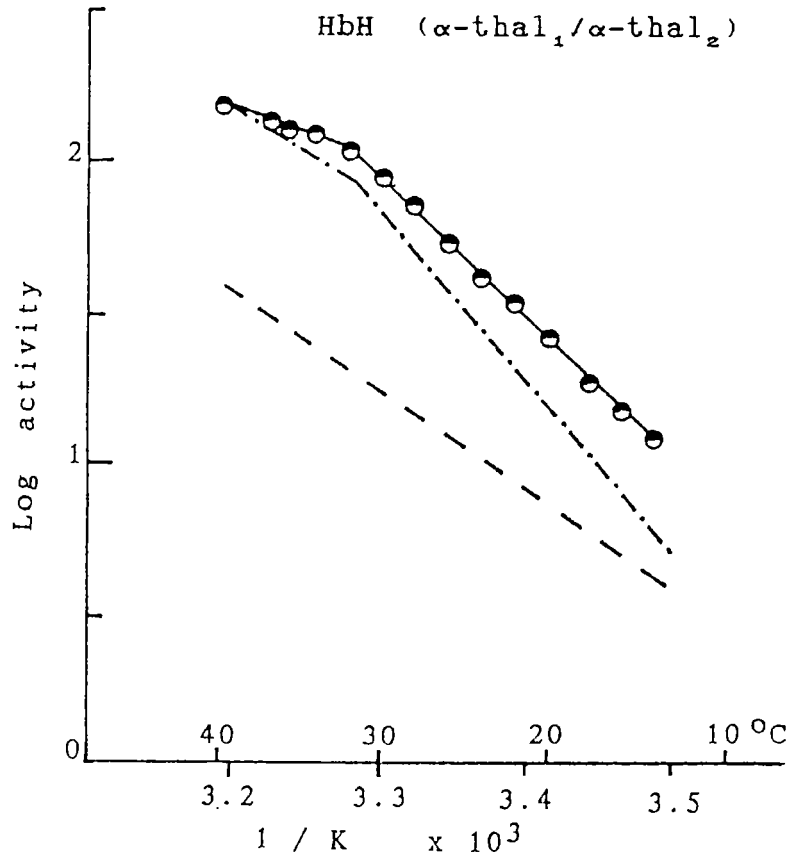


Figure 23: The Arrhenius profiles of erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase from HbH (α-thal 1/α-thal 2) disease. Membranes were prepared as described in "Materials and Methods". The enzyme activities were assayed by PK/LDH coupled enzyme method. The specific activity was μ mole ATP/ μ mole phospholipids/minute: ·-· and --- represent normal control with and without addition of calmodulin, respectively; -●- and -o- represent thalassemia samples with and without addition of calmodulin, respectively.

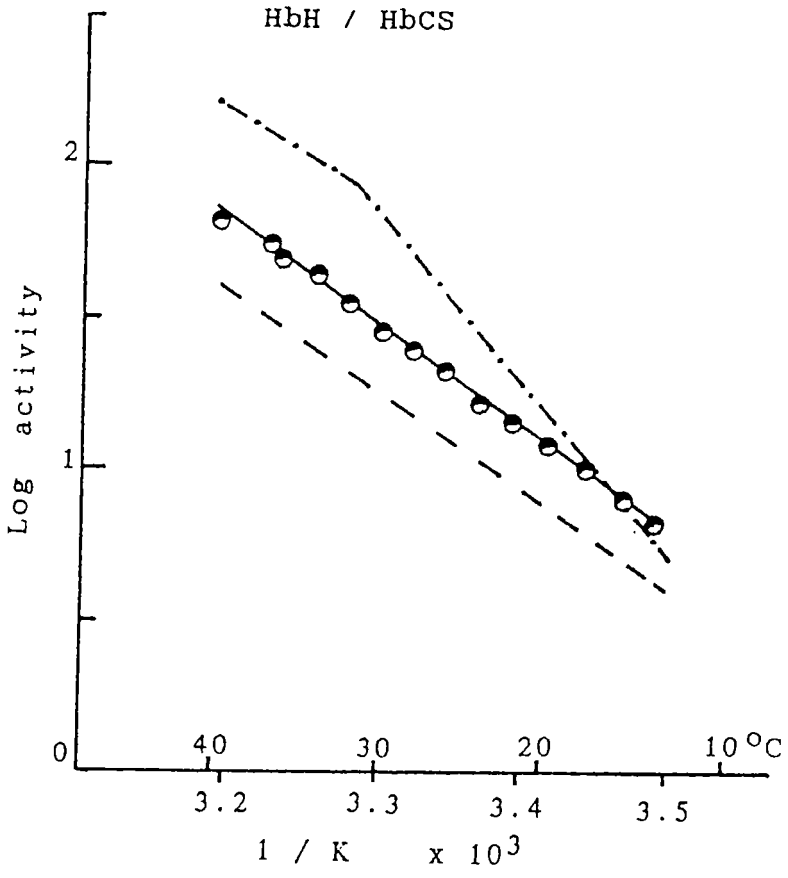


Figure 24: The Arrhenius profiles of erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase from HbH/HbCS disease. Membranes were prepared as described in "Materials and Methods". The enzyme activities were assayed by PK/LDH coupled enzyme method. The specific activity was μ mole ATP/ μ mole phospholipids/ minute: ·-· and --- represent normal control with and without addition of calmodulin, respectively; -o- and -o- represent thalassemia samples with and without addition of calmodulin, respectively.

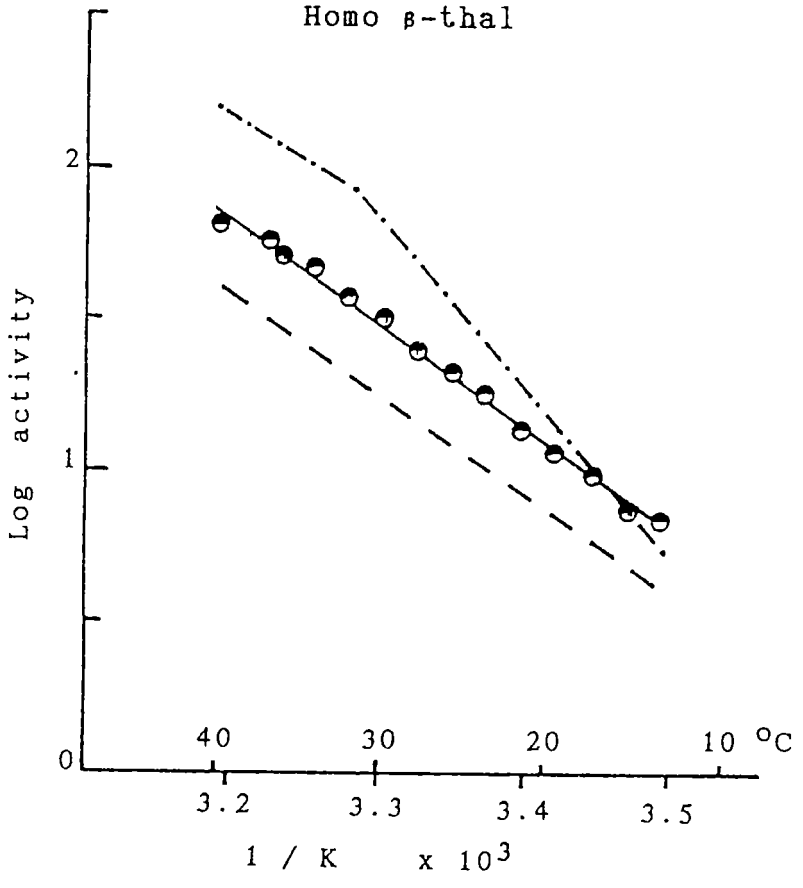


Figure 25: The Arrhenius profiles of erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase from homozygous β-thalasseimia disease. Membranes were prepared as described in "Materials and Methods". The enzyme activities were assayed by PK/LDH coupled enzyme method. The specific activity was μ mole ATP/ μ mole phospholipids/minute: ·-· and --- represent normal control with and without addition of calmodulin, respectively; -●- and -o- represent thalassemia samples with and without addition of calmodulin, respectively.

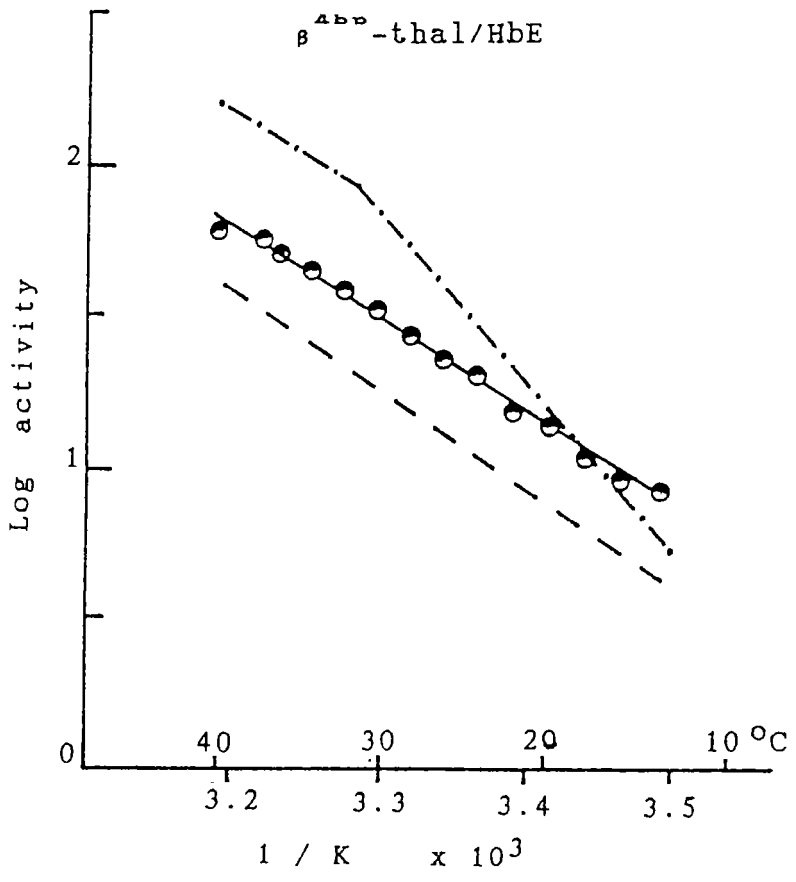


Figure 26: The Arrhenius profiles of erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase from β-thalassemia (4 bp deletion)/HbE disease. Membranes were prepared as described in "Materials and Methods". The enzyme activities were assayed by PK/LDH coupled enzyme method. The specific activity was μ mole ATP/ μ mole phospholipids/minute: •-• and --- represent normal control with and without addition of calmodulin, respectively; -●- and -o- represent thalassemia samples with and without addition of calmodulin, respectively.

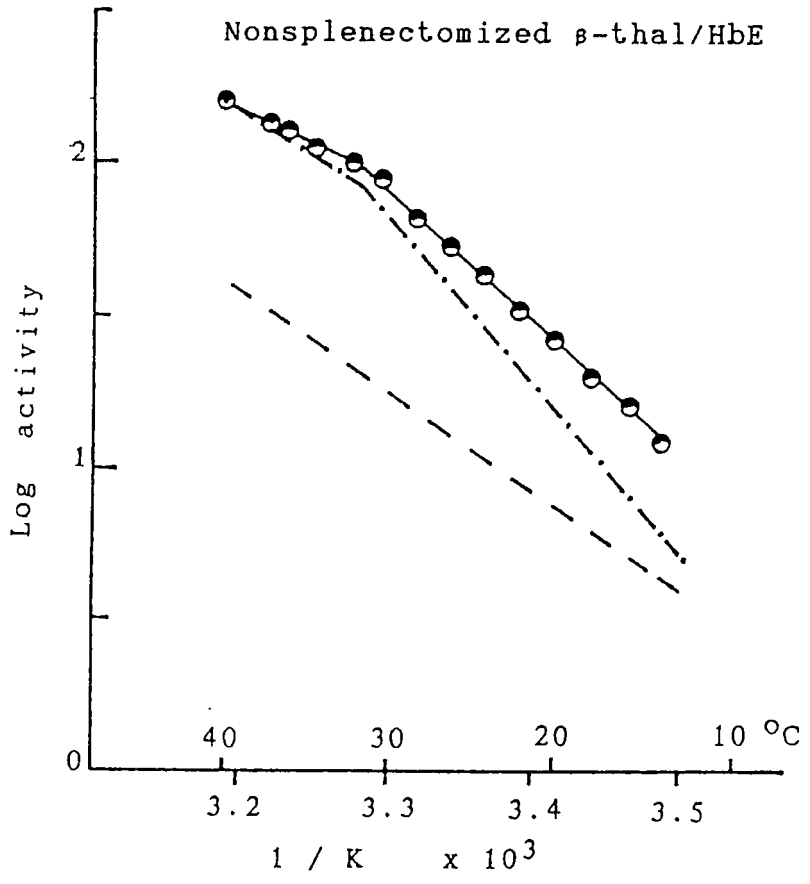


Figure 27: The Arrhenius profiles of erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase from nonsplenectomized β-thalassemia/ HbE disease. Membranes were prepared as described in "Materials and Methods". The enzyme activities were assayed by PK/LDH coupled enzyme method. The specific activity was μ mole ATP/ μ mole phospholipids/minute: •-• and --- represent normal control with and without addition of calmodulin, respectively; -●- and -o- represent thalassemia samples with and without addition of calmodulin, respectively.

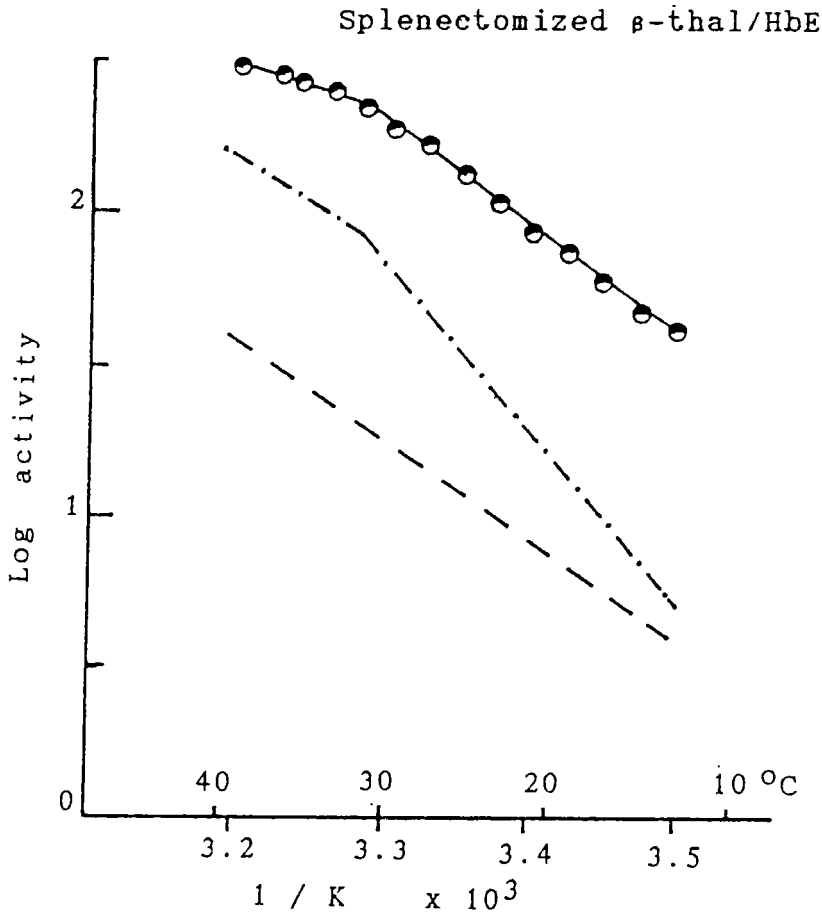


Figure 28: The Arrhenius profiles of erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase from splenectomized β-thalassemia /HbE disease. Membranes were prepared as described in "Materials and Methods". The enzyme activities were assayed by PK/LDH coupled enzyme method. The specific activity was μ mole ATP/ μ mole phospholipids/minute: •-• and --- represent normal control with and without addition of calmodulin, respectively; -●- and -o- represent thalassemia samples with and without addition of calmodulin, respectively.

decoupling of the ATPase and the Ca^{2+} translocation activities of the enzyme.

4. The Effects of Calmodulin-Binding Domain on the Arrhenius Properties of the Ca^{2+}/Mg^{2+}-ATPase

To investigate the effect of calmodulin-binding domain on the alterations of the Arrhenius properties of the enzyme, normal erythrocyte membrane preparations were treated with trypsin in order toremove the domain as described under "Materials and Methods". The trypsinized membranes were then assayed for the Ca^{2+}/Mg^{2+}-ATPase activity, both in the absence and presence of bovine brain calmodulin. The Arrhenius profiles of the trypsin-treated enzyme are shown in Figure 29. The profiles were not different between assays with and without addition of the calmodulin indicating that trypsin treatment had indeed removed the calmodulin-binding domain. However, the specific activity was somewhat lower than the activated preparation which might be due to damage to other domains of the enzyme. The profile also showed a break at 32 °C. The values of activation energy, both below and above the Arrhenius break, were similar to those of the normal enzyme with calmodulin activation (Table 6).

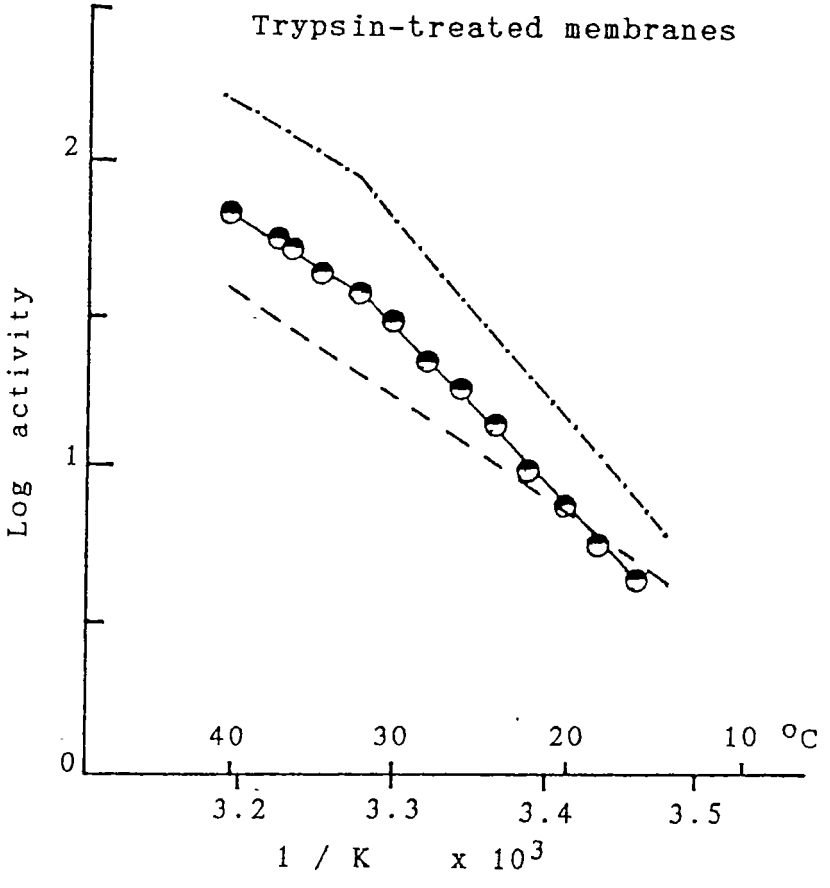


Figure 29: The Arrhenius profiles of Ca^{2+}/Mg^{2+}-ATPase from normal erythrocyte membranes treated with trypsin. Membranes were incubated with trypsin and prepared as described in "Materials and Methods". The enzyme activities were assayed by PK/LDH coupled enzyme method. The specific activity was μ mole ATP/ μ mole phospholipids/minute: •-• and --- represent normal control with and without addition of calmodulin, respectively; -●- and -o- represent thalassemia samples with and without addition of calmodulin, respectively.

5. Studies of the Arrhenius Properties of the Ca^{2+}/Mg^{2+}-ATPase in Oxidised Erythrocytes

The effects of oxidative conditions on the activity of the Ca^{2+}/Mg^{2+}-ATPase were studied. Normal human erythrocytes were treated with a number of oxidants in order to mimic the condition of oxidative stress in thalassemic erythrocytes. The calmodulin-deficient membranes were prepared as described in "Materials and Methods". The Arrhenius profiles of the Ca^{2+}/Mg^{2+}-ATPase activity were studied in order to compare with those from thalassemia samples.

The effects of diamide and p-chloromercuribenzoate (PCMB) on the enzyme activity were not obvious when the oxidants were used on intact erythrocytes. On the other hand, when the erythrocyte membrane preparations were oxidised with diamide or PCMB, alterations of the Arrhenius profiles could be observed (Figure 30 and 31, respectively). However, the changes did not resemble those from thalassemias. The profiles still showed the calmodulin response of the enzyme. Both calmodulin-unstimulated and calmodulin-stimulated ATPase activities were inhibited, and the inhibitory effect was concentration dependent (Figure 32). These experiments indicated that the formation of cross-linkage by diamide and the reduction of the sulfhydryl content by PCMB reaction could inhibit the Ca^{2+}/Mg^{2+}-ATPase activity. Both reactions could not alter the response of the enzyme to calmodulin stimulation. The experiments also indicated that the alterations

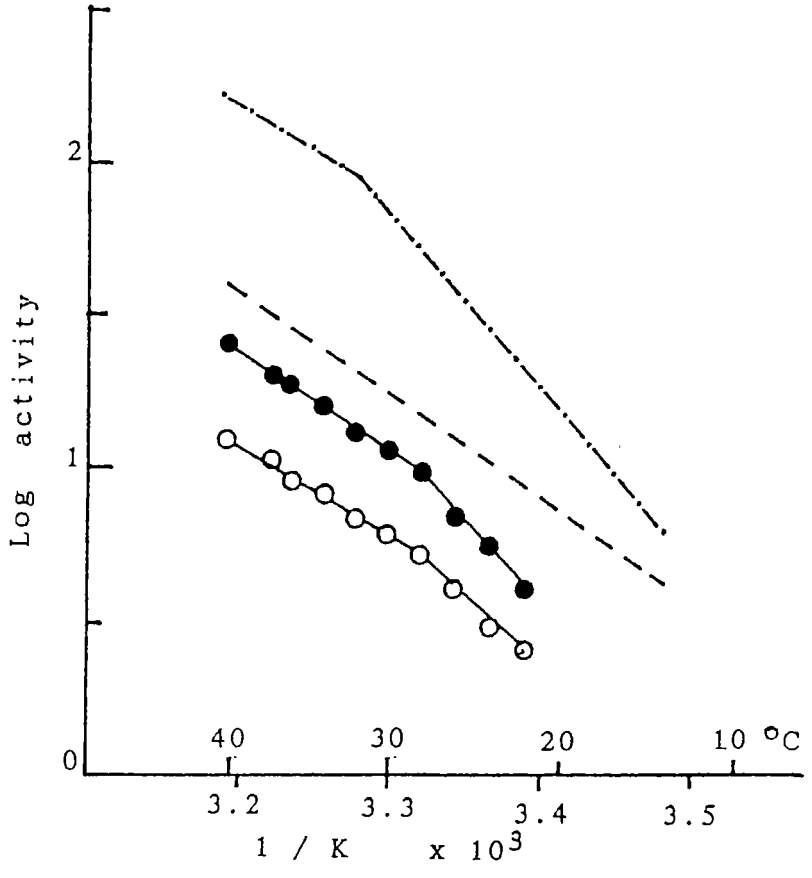


Figure 30: The Arrhenius profiles of Ca^{2+} /Mg^{2+} -ATPase from normal erythrocyte membrane treated with diamide . Normal erythrocyte membrane preparations were treated with 0.5 mM diamide at 37 °C, 15 min, prior to assay by coupled enzyme method as described in "Materials and Methods". The specific activity was μ mole ATP/ μ mole phospholipids/ minute: •-• and ---, control; -●- and -o-, treated; with and without addition of calmodulin, respectively.

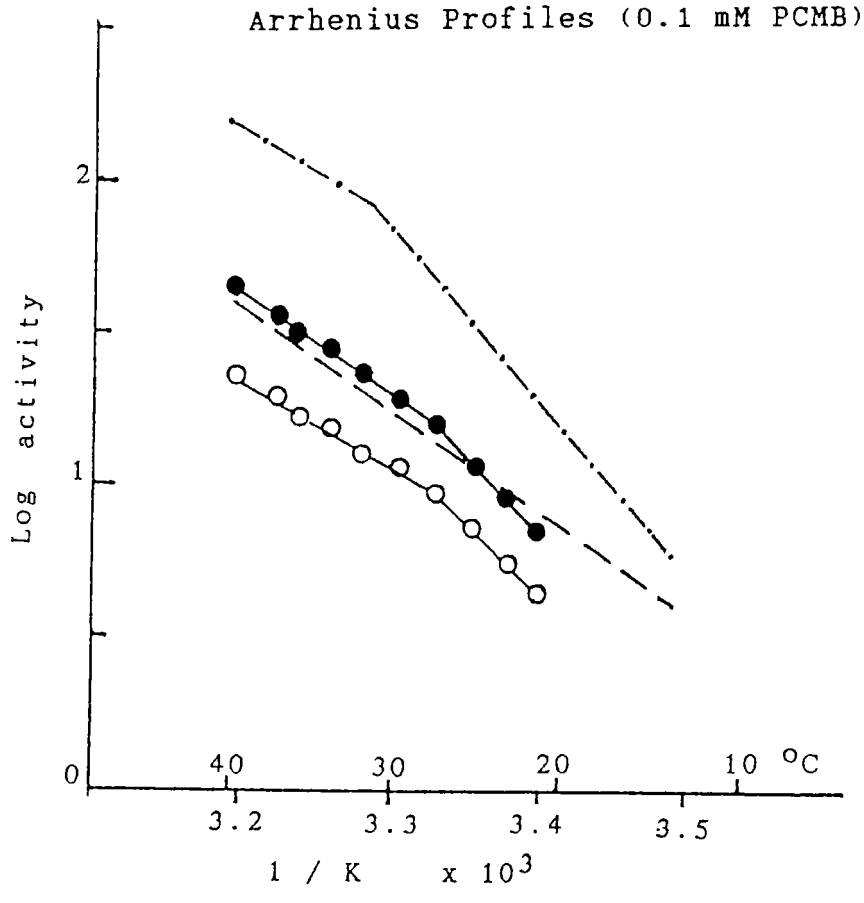


Figure 31: The Arrhenius profiles of Ca^{2+} /Mg^{2+} -ATPase from normal erythrocyte membrane treated with p-chloromercuribenzoate (PCMB). Normal erythrocyte membrane preparations were oxidised with 0.1 mM PCMB at 37 °C, for 15 min. The enzyme activity was assayed by coupled enzyme method as described in "Materials and Methods". The specific activity was μ mole ATP/μ mole phospholipids/minute: .—. and ---, control; -●- and -o-, oxidised; with and without addition of calmodulin, respectively.

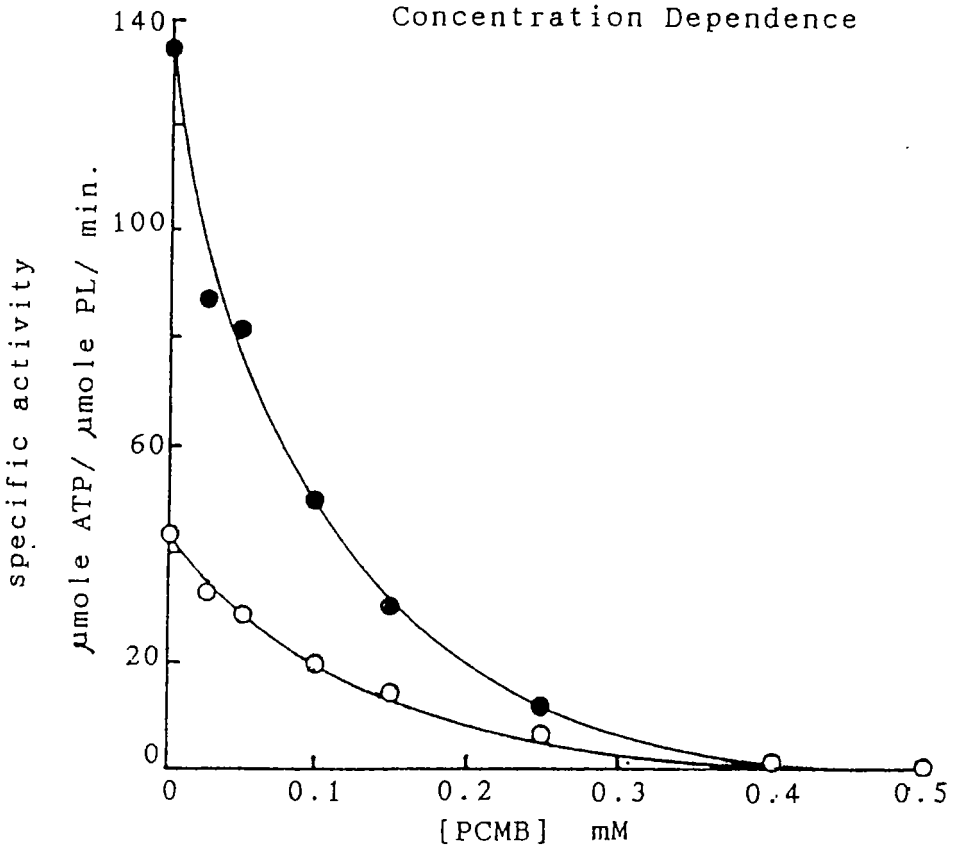


Figure 32: Effects of p-chloromercuribenzoate (PCMB) on erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase. Normal erythrocyte membrane preparations were oxidised at 37 °C, 15 min. The enzyme activity was assayed by coupled enzyme method as described in "Materials and Methods": -●- and -o-, with and without addition of calmodulin, respectively.

of the Ca^{2+}/Mg^{2+} -ATPase activities in thalassemia samples were unlikely to be the effects of formation of cross-linkage and reduction of sulfhydryl content in the enzyme molecule.

Other oxidants which are known to generate oxygen free-radicals were used to treat normal human erythrocytes. The oxidants used included phenazine methosulfate (PMS) tert-butylhydroperoxide (TBH), and phenylhydrazine (PHZ). Figure 33 shows the Arrhenius profile of Ca^{2+}/Mg^{2+}-ATPase treated with 2 mM PMS. The enzyme did not respond to calmodulin activation. Indeed, the activity was already activated. The discontinuity of the profile disappeared. The alterations were resemble those from thalassemic samples. Figure 34 shows the concentration-dependence curve of the effect of phenazine methosulfate on Ca^{2+}/Mg^{2+}-ATPase. The response to calmodulin stimulation was decreased rapidly as the concentration of PMS was increased. The calmodulin-activated ATPase activity was decreased as the concentration of PMS lower than 1 mM and became increasing again as the concentration of the oxidant were increased. However, an increase in the calmodulin-activated Ca^{2+}-ATPase activity were observed in accordant to the increases in Ca^{2+} and calmodulin-independent ATPase activities.

The effects of 2 mM TBH on the Arrhenius profile of Ca^{2+}/Mg^{2+}-ATPase are shown in Figure 35. The oxidant caused reduction in both the discontinuity of the profile and the calmodulin-stimulating effect.

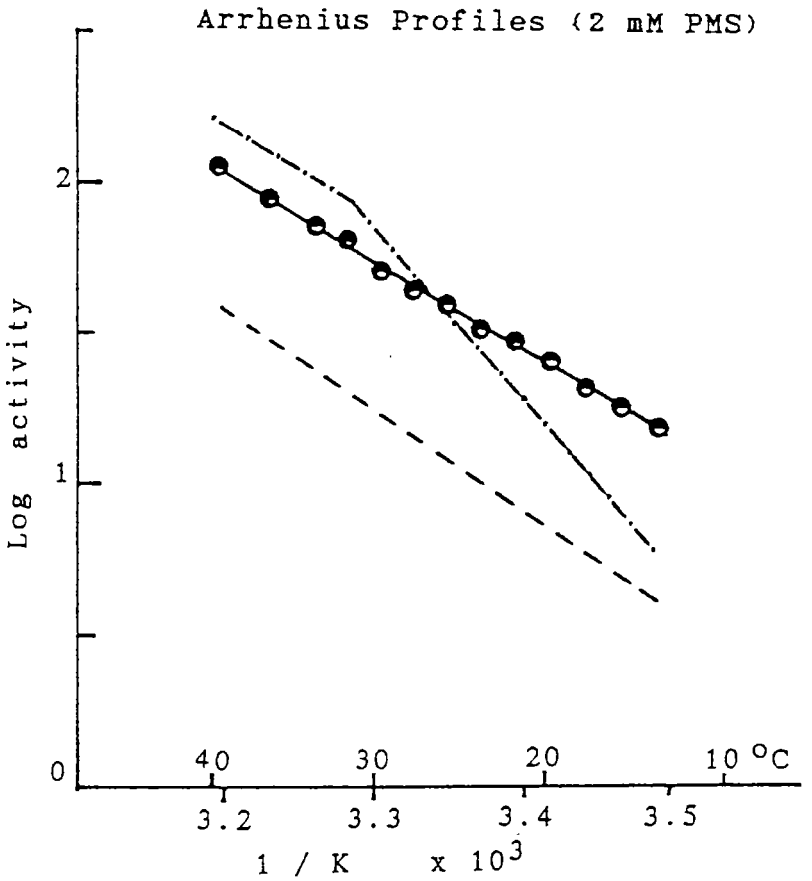


Figure 33: The Arrhenius profiles of Ca^{2+} /Mg^{2+} -ATPase from normal erythrocyte membrane treated with phenazine methosulfate (PMS). Normal human erythrocytes were oxidised with 2 mM PMS at 37 °C, for 1 hr. The membranes were prepared and the enzyme activity was assayed by coupled enzyme method as described in "Materials and Methods". The specific activity was µmole ATP/µ mole phospholipids/minute: ._. and ---, control; -●- and -o-, oxidised; with and without addition of calmodulin, respectively.

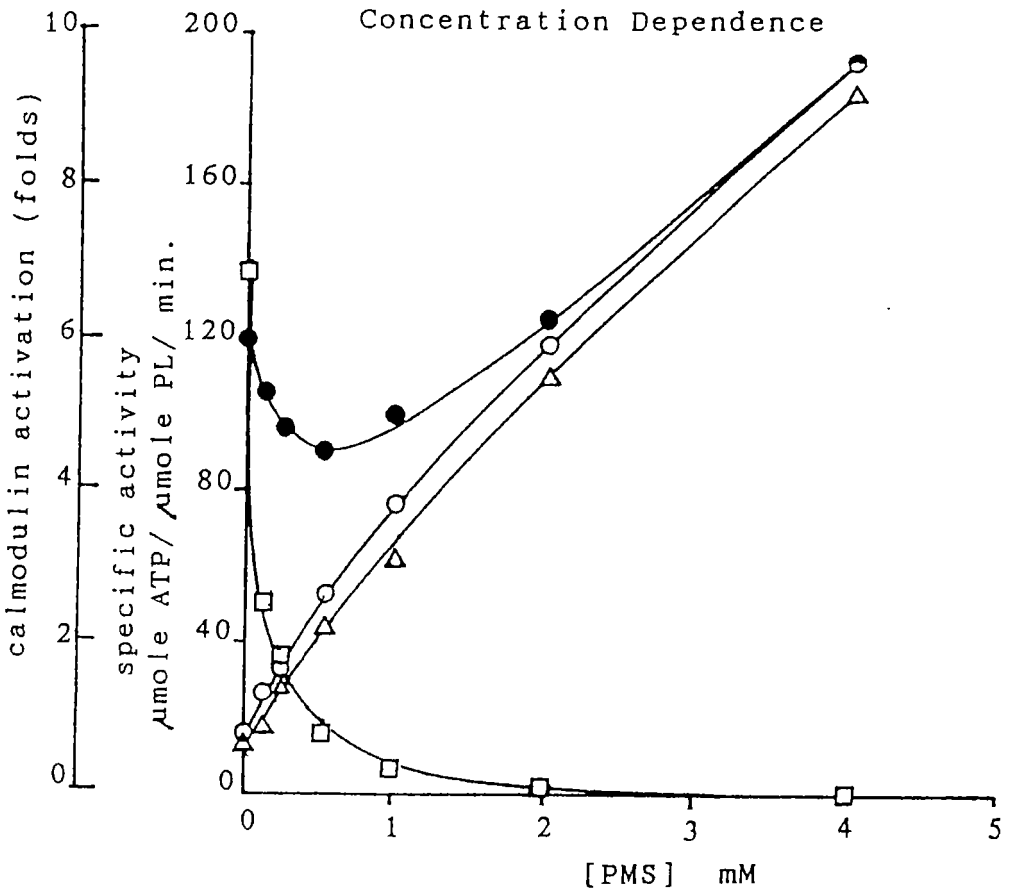


Figure 34: Effects of phenazine methosulfate (PMS) on erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase. Normal human erythrocytes were oxidised at 37 °C, 1 h. The membranes were prepared and the enzyme activity was assayed as described in "Materials and Methods". The enzyme assay conditions were: Ca^{2+} ions present but with, -●-, and without, -o-,addition of calmodulin; -▼-, absent of both Ca^{2+} ions and calmodulin; -□-, calmodulin activation.

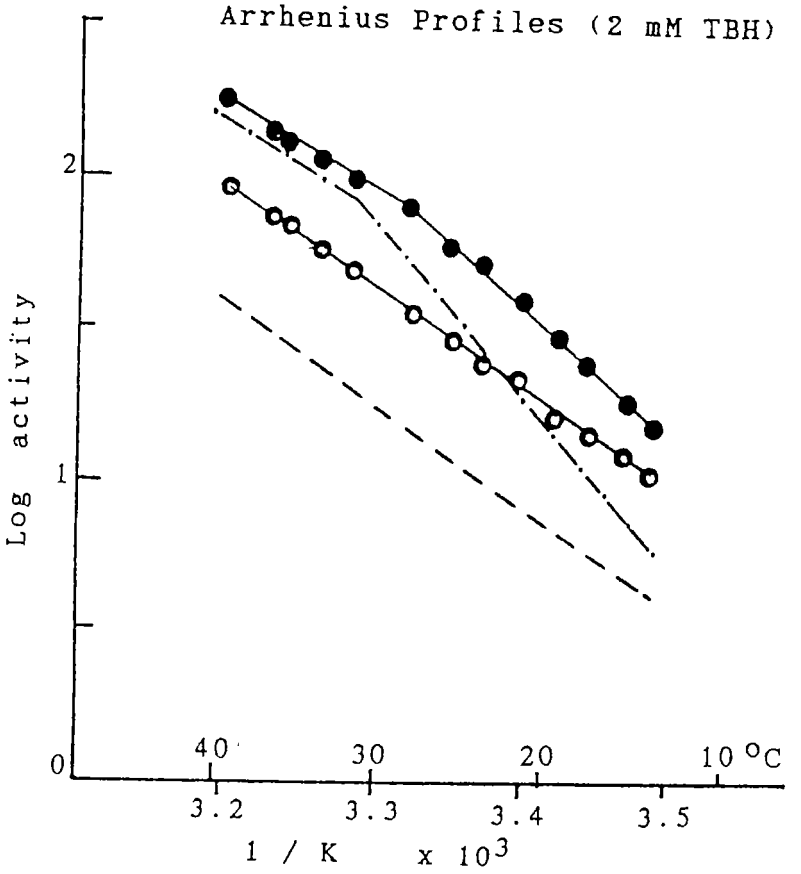


Figure 35: The Arrhenius profiles of Ca^{2+} /Mg^{2+} -ATPase from normal erythrocyte membrane treated with tert-butylhydroperoxide (TBH). Normal human erythrocytes were oxidised with 2 mM TBH at 37 °C, 1 hr. Membranes were prepared and the enzyme activity was assayed by coupled enzyme method as described in "Materials and Methods". The specific activity was μ mole ATP/μ mole phospholipids/ minute: ._. and ---, control; -●- and -o-, oxidised; with and without addition of calmodulin, respectively.

Figure 36 shows that TBH inhibited the Ca^{2+}/calmodulin-stimulated ATPase at concentration lower than 1 mM. As similar to the effect of phenazine methosulfate, higher concentrations of TBH caused an increase in the Ca^{2+}/calmodulin-stimulated ATPase activity in accordant to an increase in the Ca^{2+}/calmodulin-independent ATPase activity. Also, the calmodulin-stimulating effect was decreased.

Figure 37 shows the effect of 1 mM phenylhydrazine on the Arrhenius profile of Ca^{2+}/Mg^{2+}-ATPase, and Figure 38 shows the concentration-dependent curve of the changes in the Ca^{2+}/calmodulin-stimulated activity, the Ca^{2+}/calmodulin-independent activity, and the calmodulin-activation effect. The results were very similar to those observed from TBH experiments, i.e. the calmodulin-stimulating effect was reduced, the discontinuity at 32 °C was disappeared, a reduction in the energy of activation at low temperature, and an increase in the Ca^{2+}/calmodulin-stimulated activity in accordant to an increase in the Ca^{2+}/calmodulin-independent ATPase.

The effects of the oxidants phenazine methosulfate, tert-butylhydroperoxide and phenylhydrazine on changes in the Arrhenius properties of the Ca^{2+}/Mg^{2+}-ATPase were very similar to those observed in the thalassemia samples. These experiments indicated that oxygen free-radical generating oxidants could interfere with the calmodulin stimulation effect and cause decoupling of the ATPase and the Ca^{2+}-translocation activities of the erythrocyte membrane

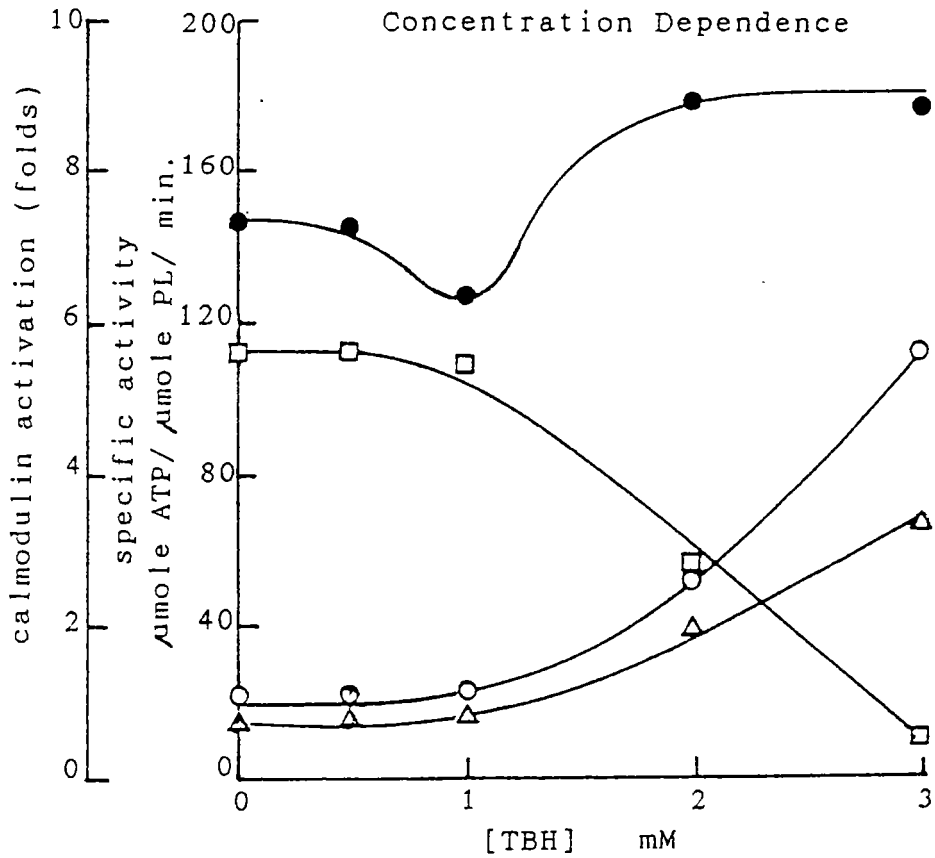


Figure 36: Effects of tert-butylhydroperoxide (TBH) on erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase. Normal human erythrocytes were oxidised at 37 °C, 1 h. The membranes were prepared and the enzyme activity was assayed as described in "Materials and Methods". The enzyme assay conditions were: Ca^{2+} ions present but with, -●-, and without, -o-,addition of calmodulin; -▼-, absent of both Ca^{2+} ions and calmodulin; -□-, calmodulin activation.

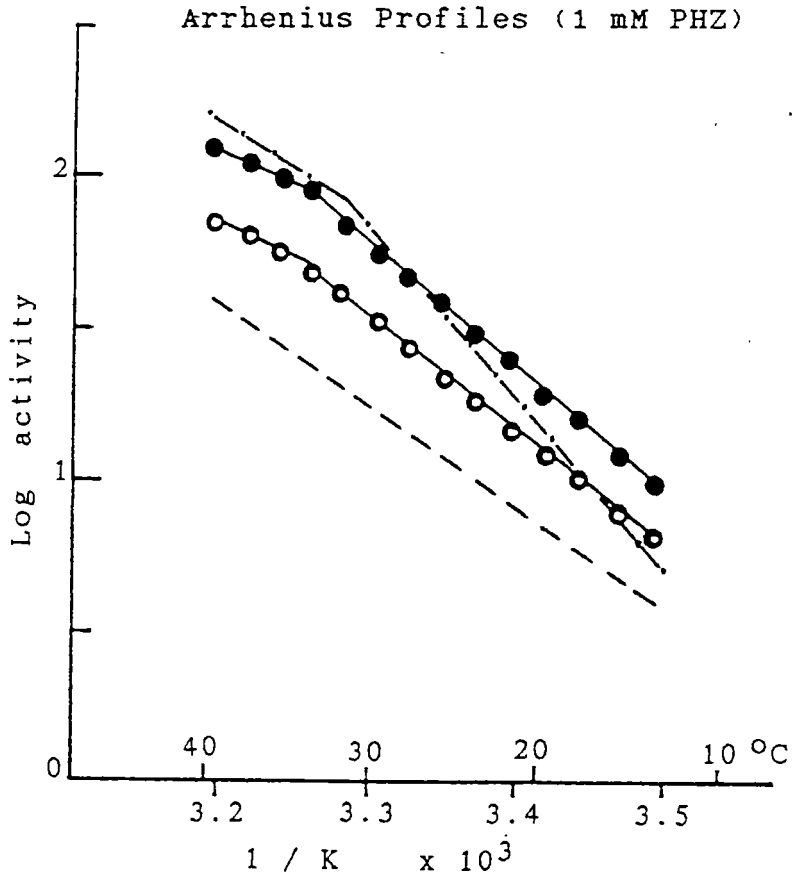


Figure 37: The Arrhenius profiles of Ca^{2+} /Mg^{2+} -ATPase from normal erythrocyte membrane treated with phenylhydrazine (PHZ). Normal human erythrocytes were oxidised with 1 mM PHZ at 37 °C, 1 hr. Membranes were prepared and the enzyme activity was assayed by coupled enzyme method as described in "Materials and Methods". The specific activity was μ mole ATP/μ mole phospholipids/ minute: ._. and ---, control; -●- and -o-, oxidised; with and without addition of calmodulin, respectively.

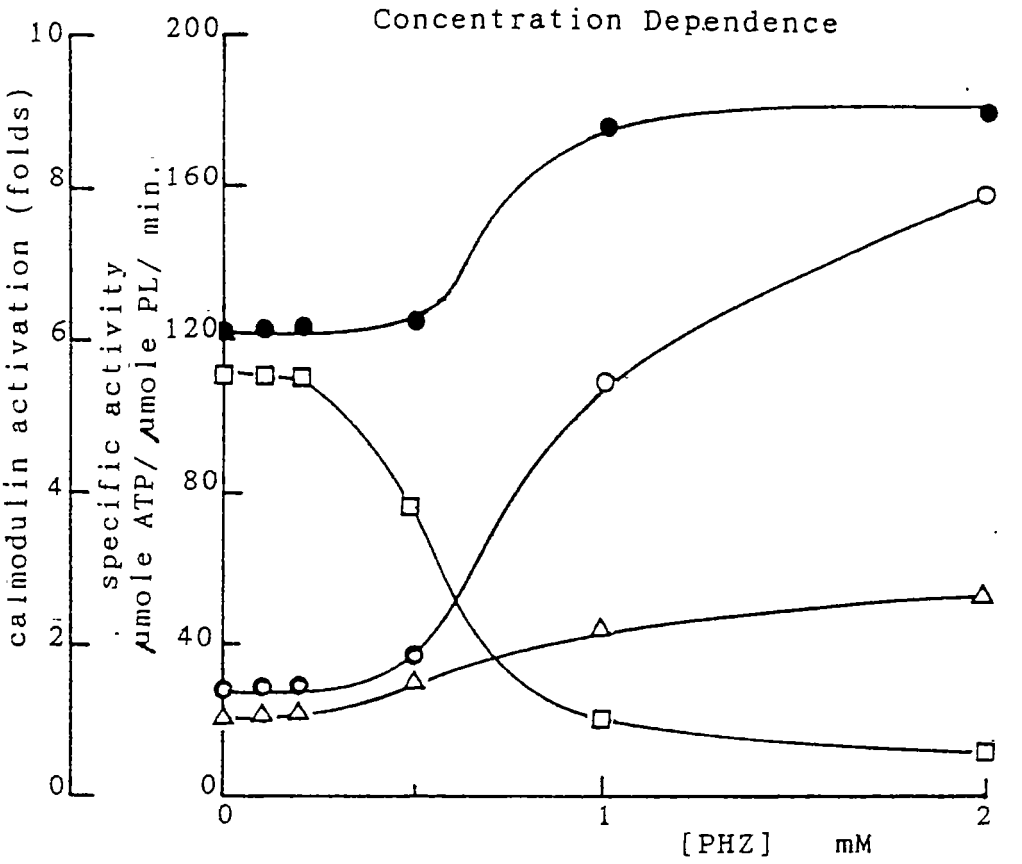


Figure 38: Effects of phenylhydrazine (PHZ) on erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase. Normal human erythrocytes were oxidised at 37 °C, 1 h. The membranes were prepared and the enzyme activity was assayed as described in "Materials and Methods". The enzyme assay conditions were: Ca^{2+} ions present but with, -●-, and without, -o-, addition of calmodulin; -▽-, absent of both Ca^{2+} ions and calmodulin; -□-, calmodulin activation.

calcium pump. Experiments on calmodulin affinity chromatography indicated that the enzyme from phenazine methosulfate-treated erythrocytes completely lost its calmodulin binding property (data not shown). The results clearly indicated that oxygen free-radical-induced loss of calmodulin stimulation was due to the defect in the calmodulin binding domain. The experiments also indicated that the alterations of the Ca^{2+}/Mg^{2+}-ATPase activity in thalassemic erythrocyte membranes were due to the effects of oxygen free-radicals.

6. Studies of the Ca^{2+}-Dependence of the Ca^{2+}/Mg^{2+}-ATPase in Thalassemic Erythrocytes

In normal erythrocyte Ca^{2+}-pump, the Ca^{2+}-translocation activity is tightly coupled to the ATPase activity, i.e. the ATPase activity is normally low in the absence of Ca^{2+} ions. Thus ATP hydrolytic activity should be higher in absence of Ca^{2+}ions when the Ca^{2+} - translocation activity is decoupled from the ATPase activity. This hypothesis was examined by assaying the Ca^{2+}/Mg^{2+}-ATPase activity in erythrocyte membrane preparations from thalassemias using coupled enzyme assay medium lacking Ca^{2+} ions and with addition of various concentrations of Ca^{2+} ions. The results are shown in Figure 39-44.

In normal erythrocyte membrane preparations, the Ca^{2+}/calmodulin-independent ATPase specific activity was 16 µmole ATP/ µmole phospholipid/ minute. The

ATPase activity was low until the total Ca^{2+} concentration in the medium was greater than 0.25 mM after which the ATPase activity began increasing. In absence of bovine brain calmodulin, the maximum specific activity of the ATPase was 40 µmole ATP/ µmole phospholipid/minute, at 2.5 mM total Ca^{2+} concentration. In the presence of bovine brain calmodulin, the maximum activity of the enzyme was at total Ca^{2+} concentration about 0.6-1.25 mM where free Ca^{2+} ion concentrations were estimated to be 10-25 µM, and the ATPase activity was stimulated about 3.5 fold higher than in the absence of calmodulin (Figure 39).

In thalassemia samples, the results (Figure 40-44) were consistent with the observations in the Arrhenius studies in that the Ca^{2+}/Mg^{2+}-ATPase was no longer or less dependent on calmodulin stimulation. However, the response profiles of the thalassemic enzymes to changes in the Ca^{2+} concentration were similar to those of the normal enzyme. In all types of thalassemia samples, except for homozygous HbCS (Figure 42), the Ca^{2}/calmodulin-independent ATPase activity was increased. Red blood cells from the splenectomized β-thalassemia/HbE were fractionated by centrifugation in "Percoll" density gradient to obtain dense ($\rho > 1.090$) and light($\rho < 1.090$) cell fractions and the Ca^{2+} - dependency profile of the ATPase was studied. The results are respectively shown in Figure 45 and 46. Both cell fractions showed loss in the Ca^{2+} and calmodulin dependence. Figure 47 shows a summary of the elevation of the Ca^{2+}/calmodulin-independent ATPase activity in thalassemic membrane preparations in comparison to

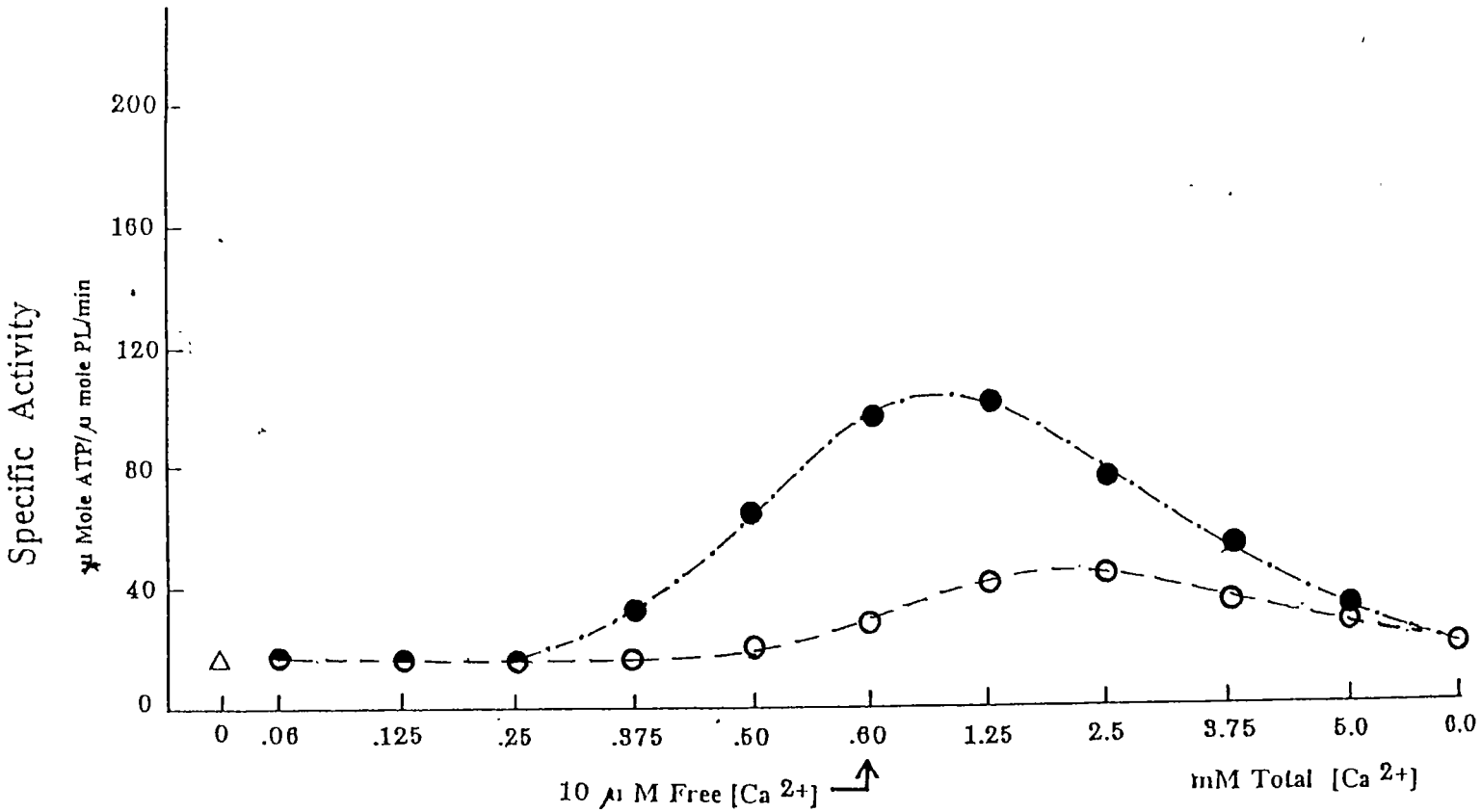


Figure 39: The response of normal erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase to changes in Ca^{2+} ions concentration. The coupled enzyme medium containing variable concentrations of Ca^{2+} ions, 1 ml, was preincubated at 37 °C, 3 min. The reaction was started by addition of 10 µl (40 µg protein) membrane suspension with, -●-, and without, -o-, addition of 1 µg bovine brain calmodulin.

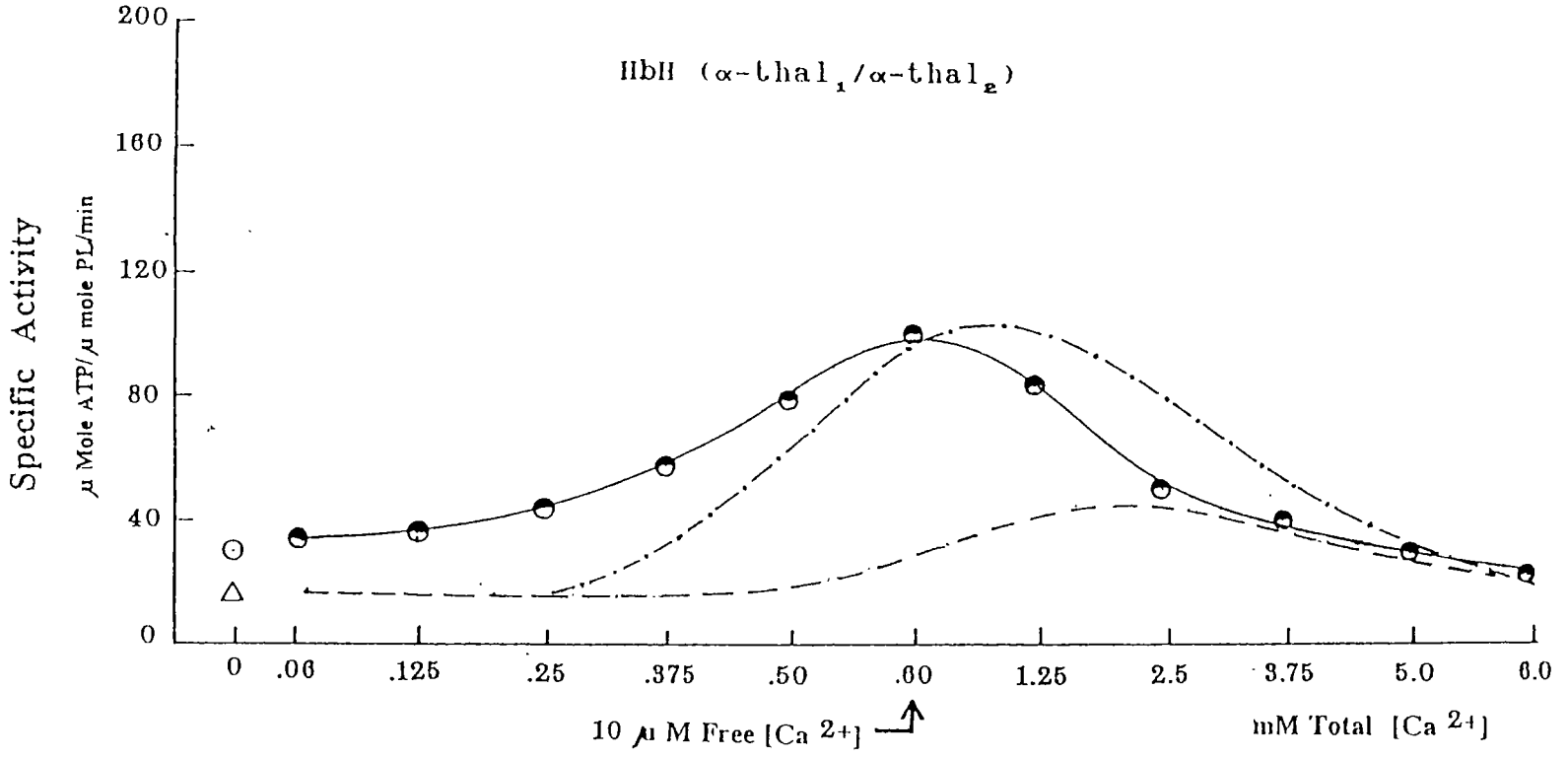


Figure 40: The response of thalassemic erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase from HbH (α-thal 1/α-thal 2) to changes in Ca^{2+} ions concentration. The coupled enzyme medium containing variable concentrations of Ca^{2+} ions, 1 ml, was preincubated at 37 °C, 3 min. The reaction was started by addition of 10 µl (40 µg protein) membrane suspension with, -●-, and without, -o-, addition of 1 µg bovine brain calmodulin; normal controls with, •-•, and without, ---, addition of calmodulin.

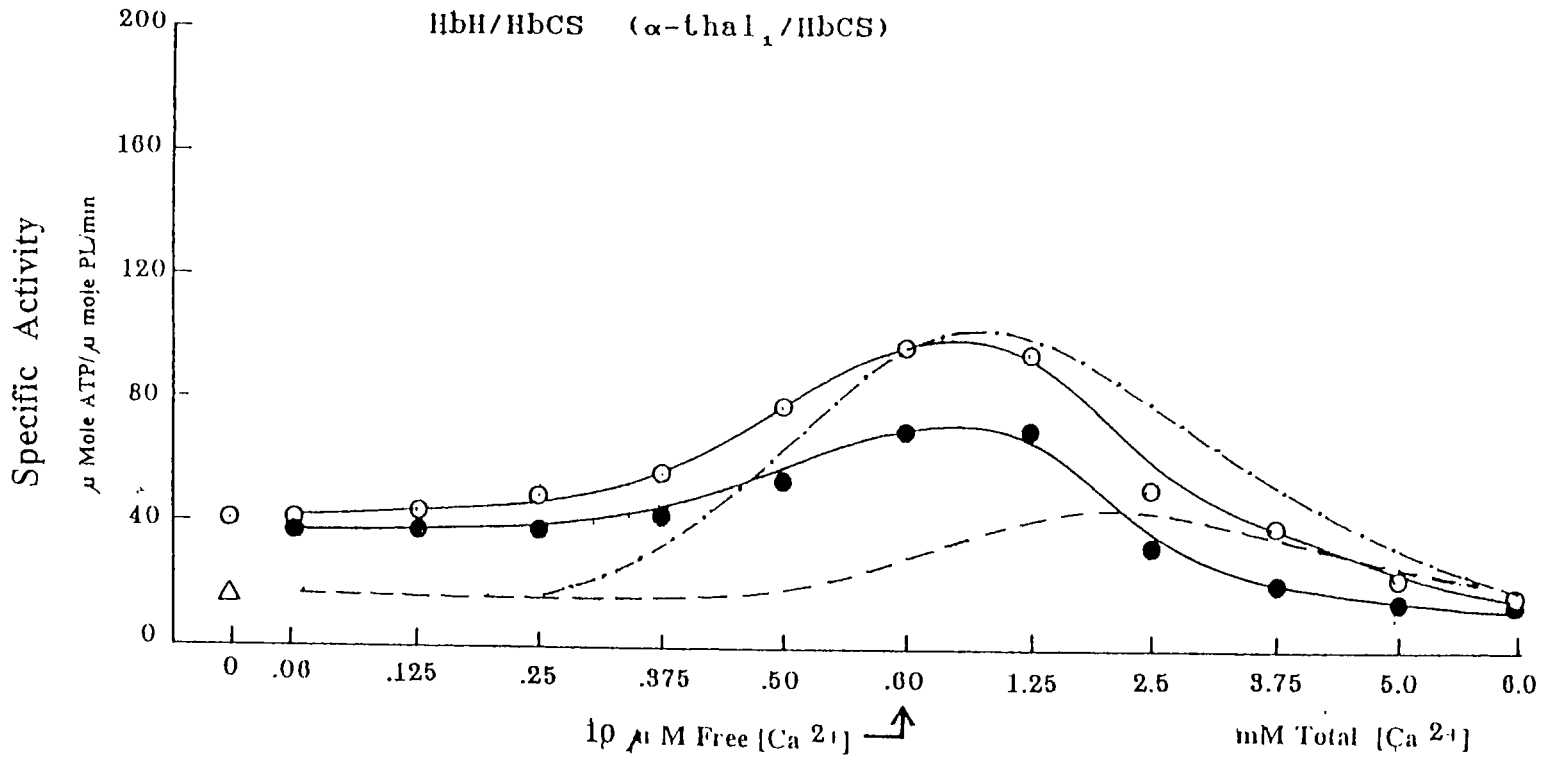


Figure 41: The response of thalassemic erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase from α-thal 1/HbCS to changes in Ca^{2+} ions concentration. The coupled enzyme medium containing variable concentrations of Ca^{2+} ions, 1 ml, was preincubated at 37 °C, 3 min. The reaction was started by addition of 10 µl (40 µg protein) membrane suspension with, -●-, and without, -o-, addition of 1 µg bovine brain calmodulin; normal controls with, .-., and without, ---, addition of calmodulin.

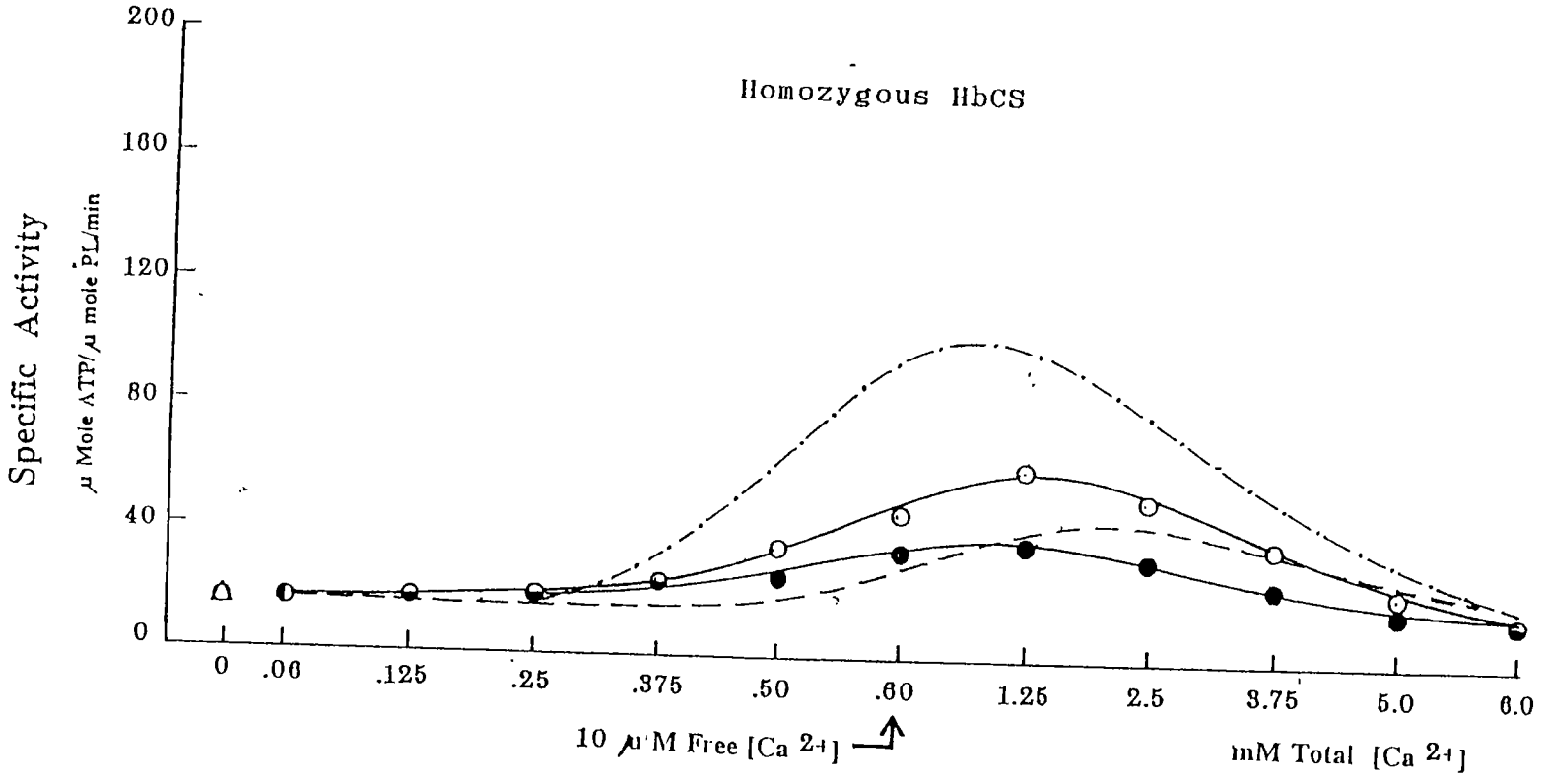


Figure 42: The response of thalassemic erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase from homozygous HbCS to changes in Ca^{2+} ions concentration. The coupled enzyme medium containing variable concentrations of Ca^{2+} ions, 1 ml, was preincubated at 37 °C, 3 min. The reaction was started by addition of 10 µl (40 µg protein) membrane suspension with, -●-, and without, -o-, addition of 1 µg bovine brain calmodulin; normal controls with, .-., and without, ---, addition of calmodulin.

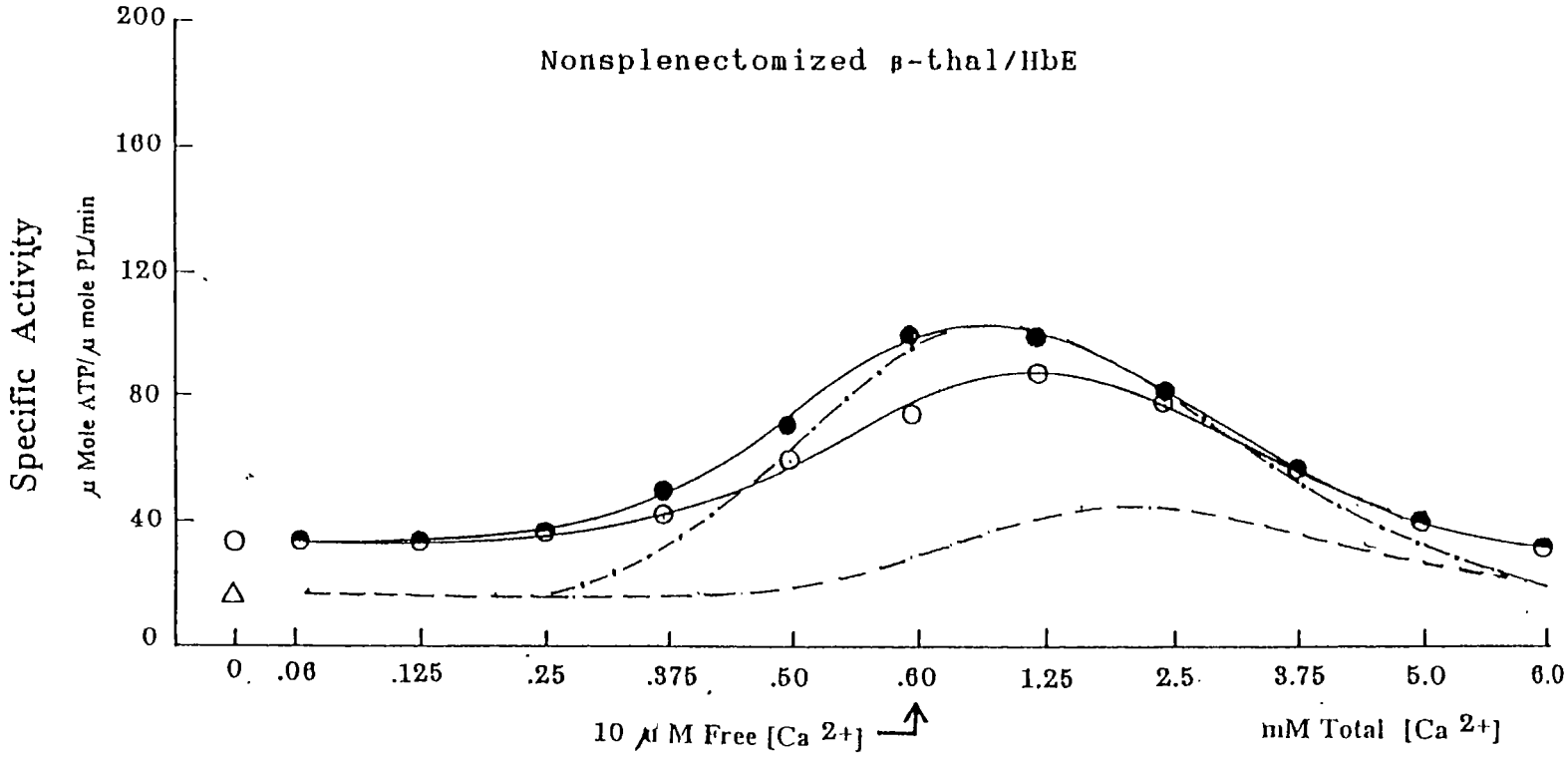


Figure 43: The response of erythrocyte membrane Ca^{2+} /Mg^{2+} - ATPase from nonsplenectomized β-thalassemia/HbE to changes in Ca^{2+} ions concentration. The coupled enzyme medium containing variable concentrations of Ca^{2+} ions, 1 ml, was preincubated at 37 °C, 3 min. The reaction was started by addition of 10 μl (40 μg protein) membrane suspension with, -●-, and without, -o-, addition of 1 μg bovine brain calmodulin; normal controls with, .-., and without, ---, addition of calmodulin.

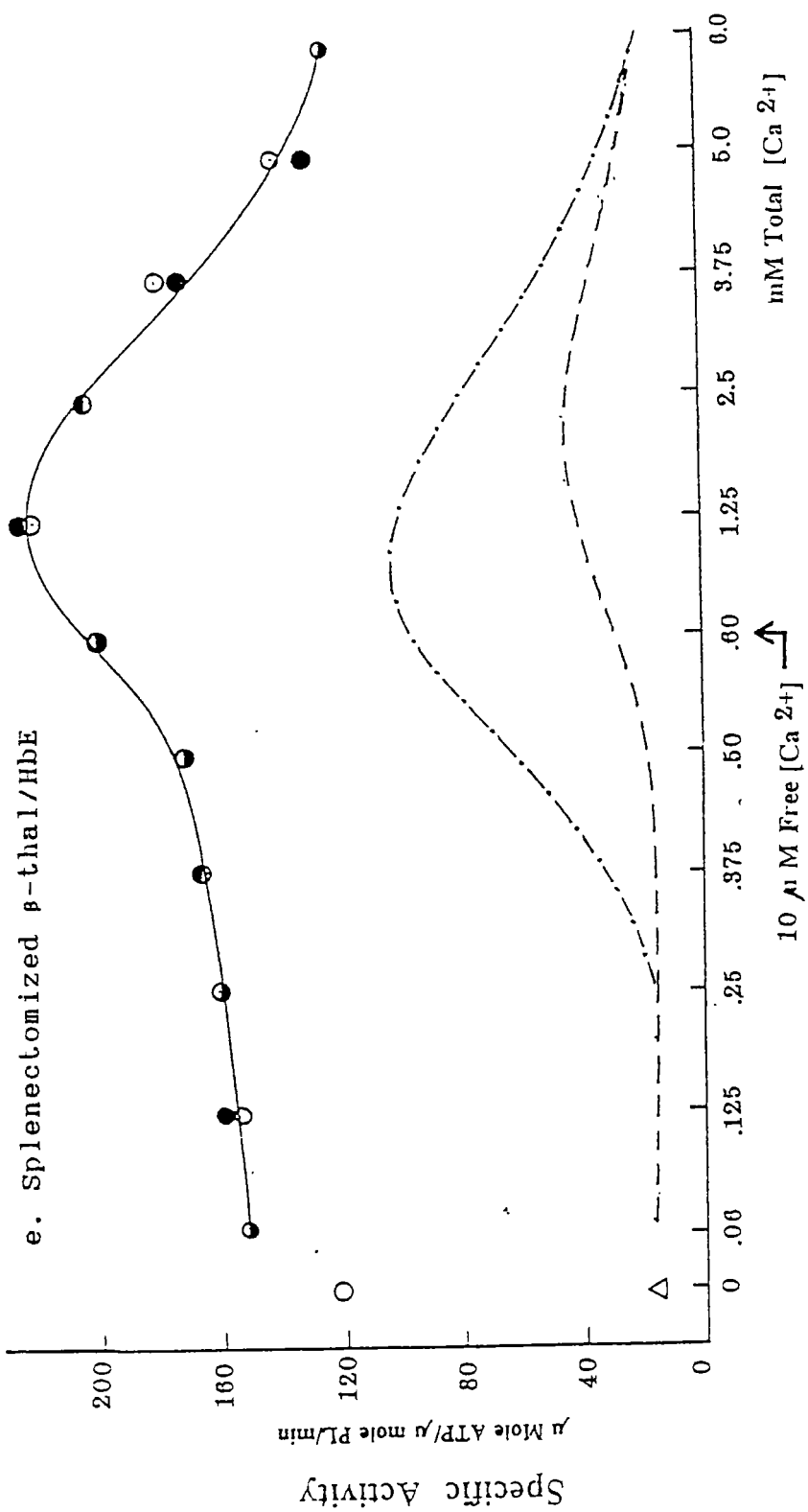


Figure 44: The response of erythrocyte membrane Ca^{2+} /Mg^{2+} - ATPase from splenectomized β-thalassemia/HbE to changes in Ca^{2+} ions concentration. The coupled enzyme medium containing variable concentrations of Ca^{2+} ions, 1 ml, was preincubated at 37 °C, 3 min. The reaction was started by addition of 10 µl (40 µg protein) membrane suspension with, -●-, and without, -o-, addition of 1 µg bovine brain calmodulin; normal controls with, .-.-, and without, ---, addition of calmodulin.

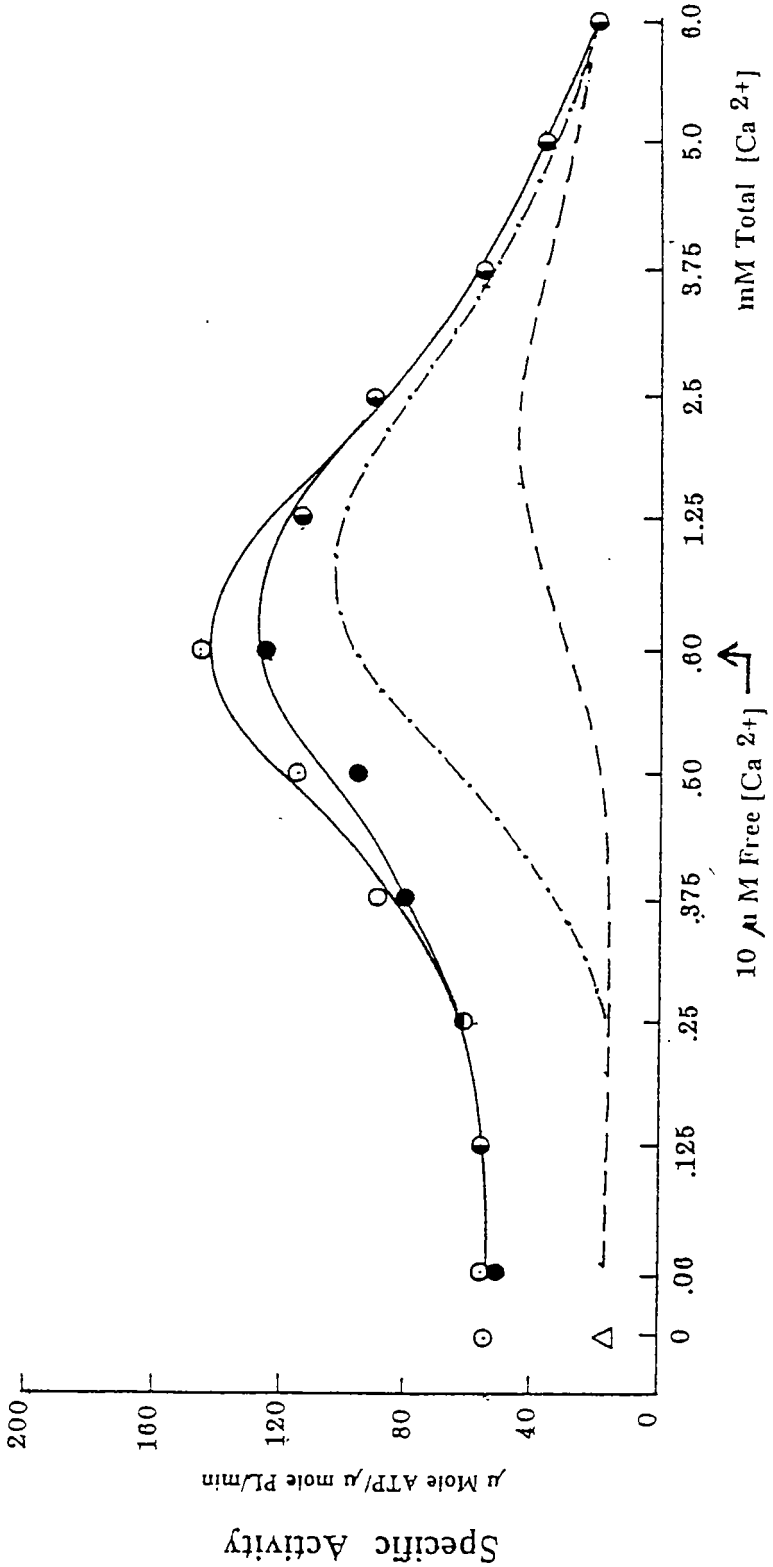


Figure 45: The response of erythrocyte membrane Ca^{2+} /Mg^{2+} - ATPase from dense cell fraction, ρ>1.090, of splenectomized β-thalassemia/HbE to changes in Ca^{2+} ions concentration. The coupled enzyme medium containing variable concentrations of Ca^{2+} ions, 1 ml, was preincubated at 37 °C, 3 min. The reaction was started by addition of 10 μl (40 μg protein) membrane suspension with, -●-, and without, -o-, addition of 1 μg bovine brain calmodulin; normal controls with, .-., and without, ---, addition of calmodulin.

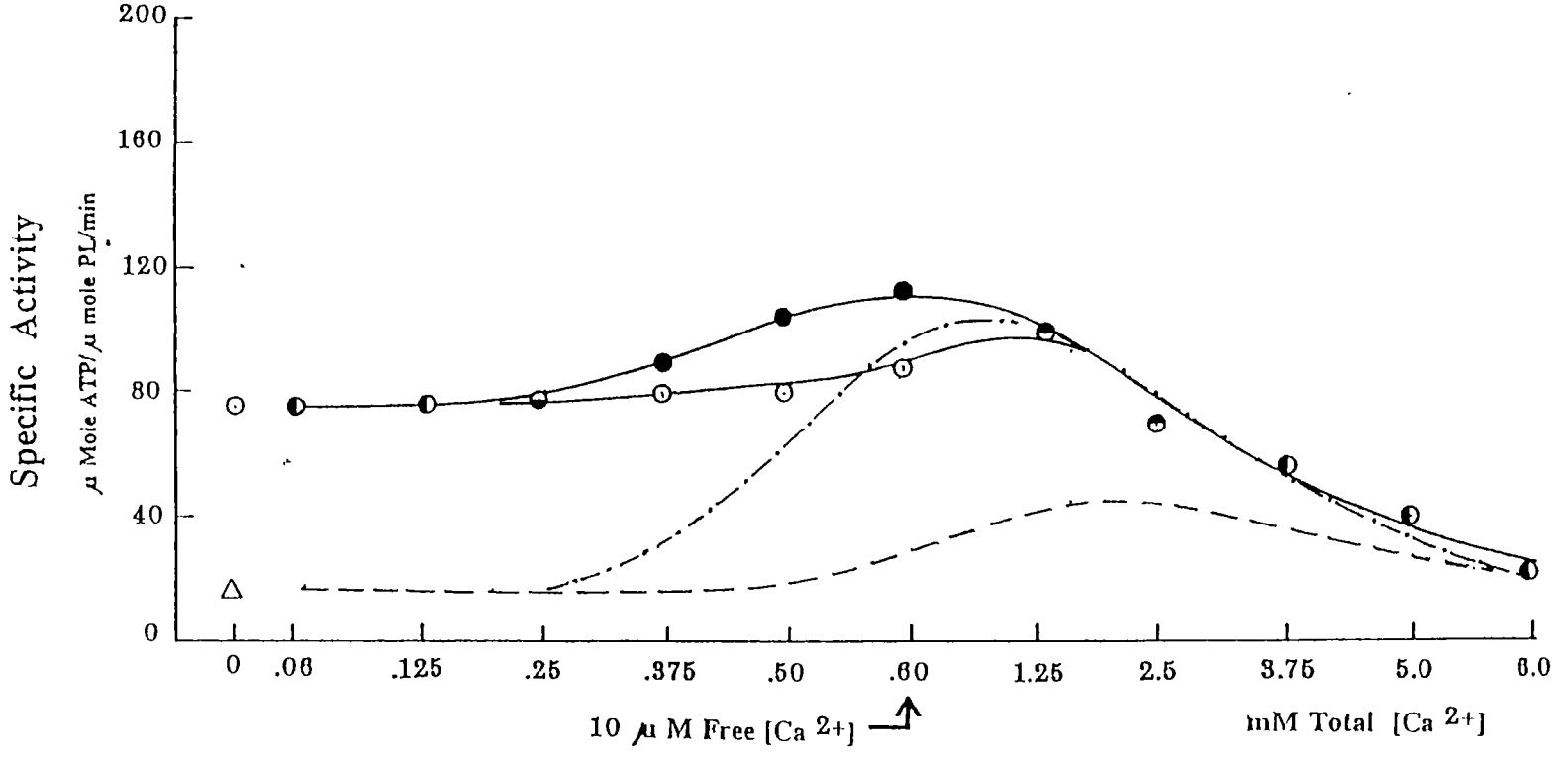


Figure 46: The response of erythrocyte membrane Ca^{2+} /Mg^{2+} - ATPase from light cell fraction, $\rho<1.090$, of splenectomized β-thalassemia/HbE to changes in Ca^{2+} ions concentration. The coupled enzyme medium containing variable concentrations of Ca^{2+} ions, 1 ml, was preincubated at 37 °C, 3 min. The reaction was started by addition of 10 µl (40 µg protein) membrane suspension with, -●-, and without, -o-, addition of 1 µg bovine brain calmodulin; normal controls with, .-., and without, ---, addition of calmodulin.

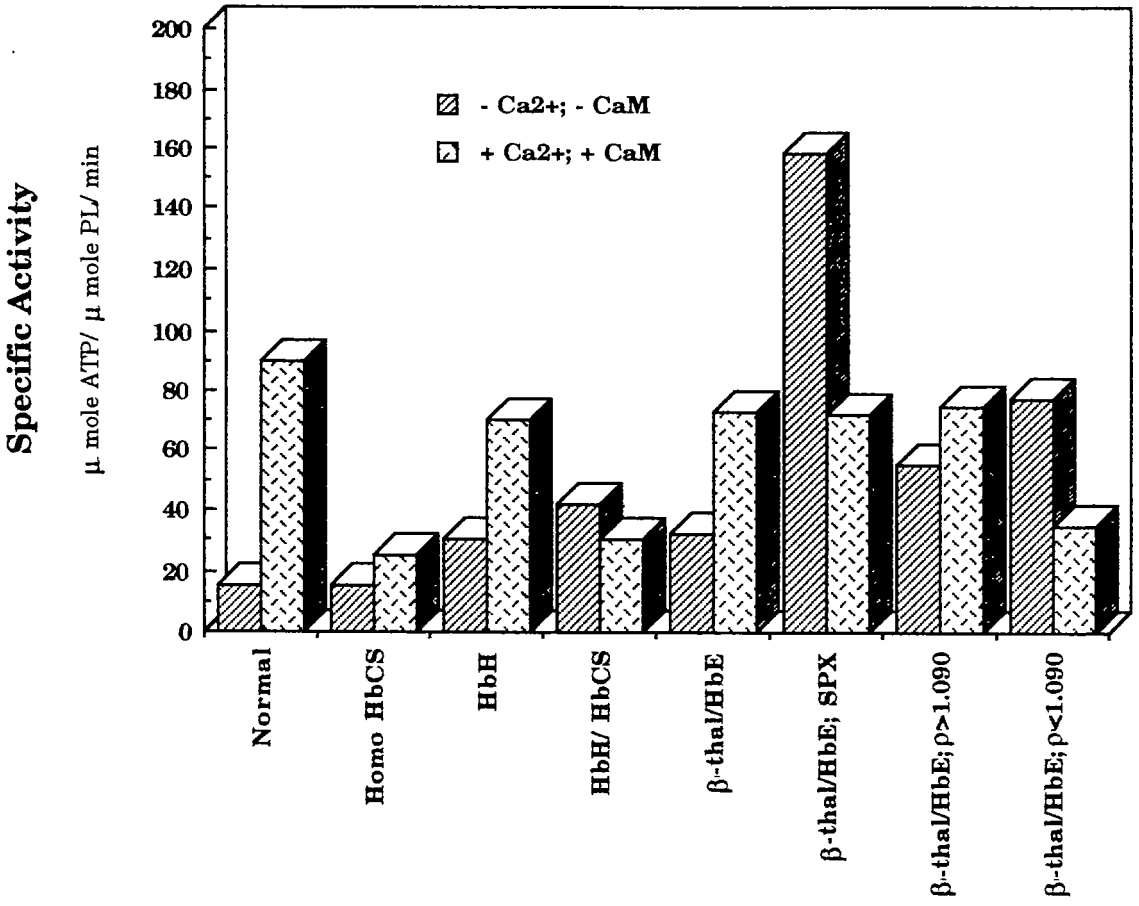


Figure 47: Specific activities of the Ca^{2+} /calmodulin-independent ATPase and the Ca2+ /calmodulin-dependent ATPase in erythrocyte membranes from various types of thalassemia.

normal samples. Figure 47 shows that the increased Ca^{2+}/calmodulin-independent ATPase activity was espectially high in samples from splenectomized β-thalassemia/HbE and the activity was higher in the light cell fraction than in the dense cell fraction. These experiments suggested that the relatively high total ATPase activities in thalassemia samples as measurable by the coupled enzyme method were due in part to elevated Ca^{2+}/calmodulin-independent ATPase activity.

7. Studies of the Ca^{2+}-Dependence of the Ca^{2+}/Mg^{2+}-ATPase in Oxidsed Erythrocytes

In order to study the effects of oxygen free-radicals on the Ca^{2+}-dependent property of the enzyme Ca^{2+}/Mg^{2+}-ATPase, intact normal human erythrocyes were incubated with oxygen free-radical generating oxidants for a given period of time. Calmodulin-deficient membranes were then prepared from the oxidised erythrocytes, and their Ca^{2+}-dependence of ATPase was studied. The results are shown in Figure 48-55.

In comparison to normal control, the oxidants phenazine methosulfate (PMS), phenylhydrazine (PHZ), tert-butylhydroperoxide (TBH), and xanthine oxidase (XO) caused alterations in the Ca^{2+}-dependent property of the Ca^{2+}/Mg^{2+}-ATPase in the manner resemble to those observed in the thalassemia samples. Consistent with the Arrhenius studies, the oxidants caused the enzyme to be independent of calmodulin activation. The oxidants also caused increase in the Ca^{2+}/calmodulin-independent

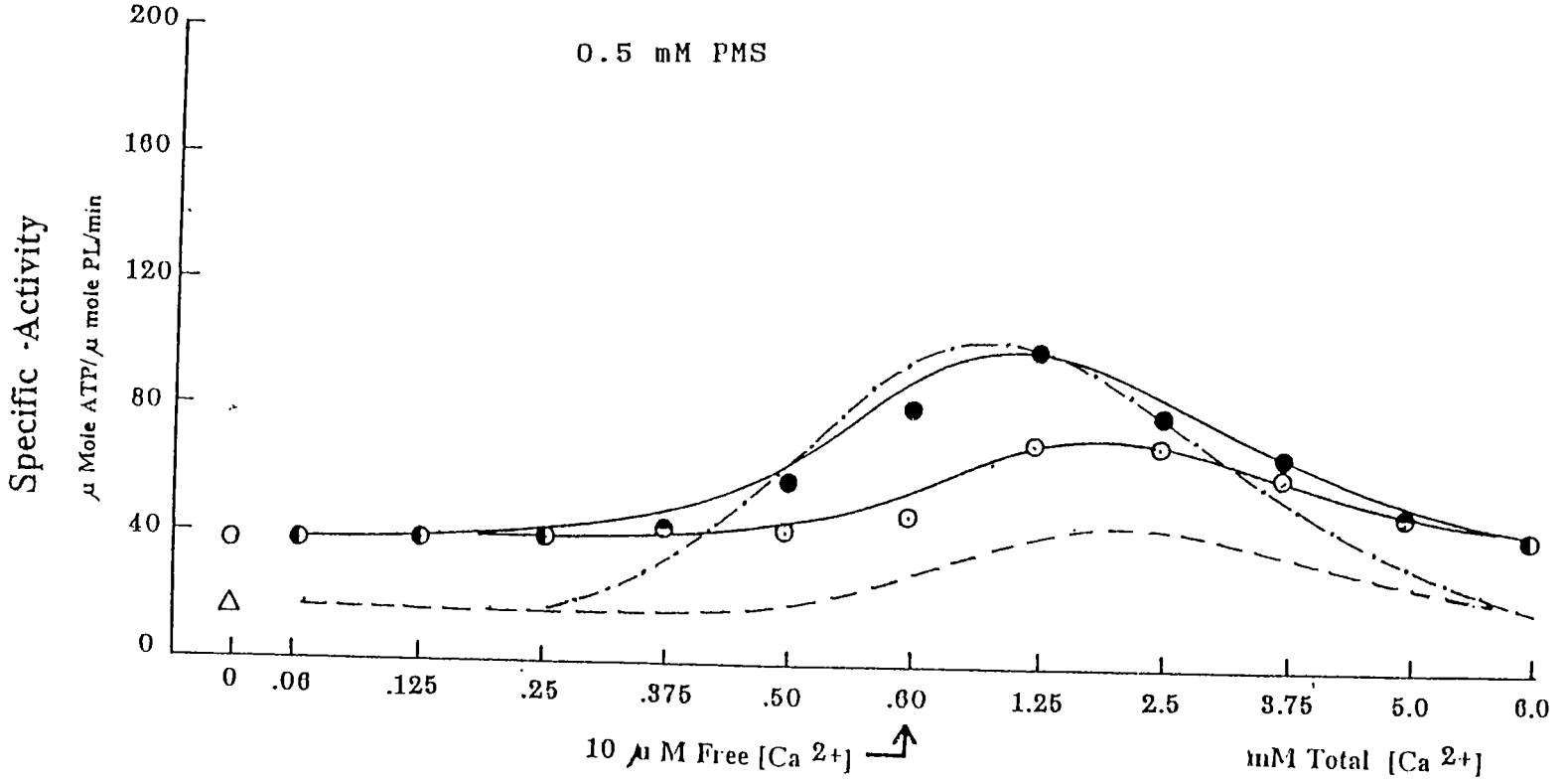


Figure 48: The responses of Ca^{2+}/Mg^{2+} -ATPase from oxidised erythrocyte membranes to changes in Ca^{2+} ions concentration. Intact normal human erythrocytes were treated with 0.5 mM phenazine methosulfate at 37 °C, 1 hr, and membranes were prepared as described in "Materials and Methods". The enzyme activity was assayed by coupled enzyme method with, -o-, and without, -o-, addition of calmodulin; normal controls with,.-., and without,---, addition of calmodulin.

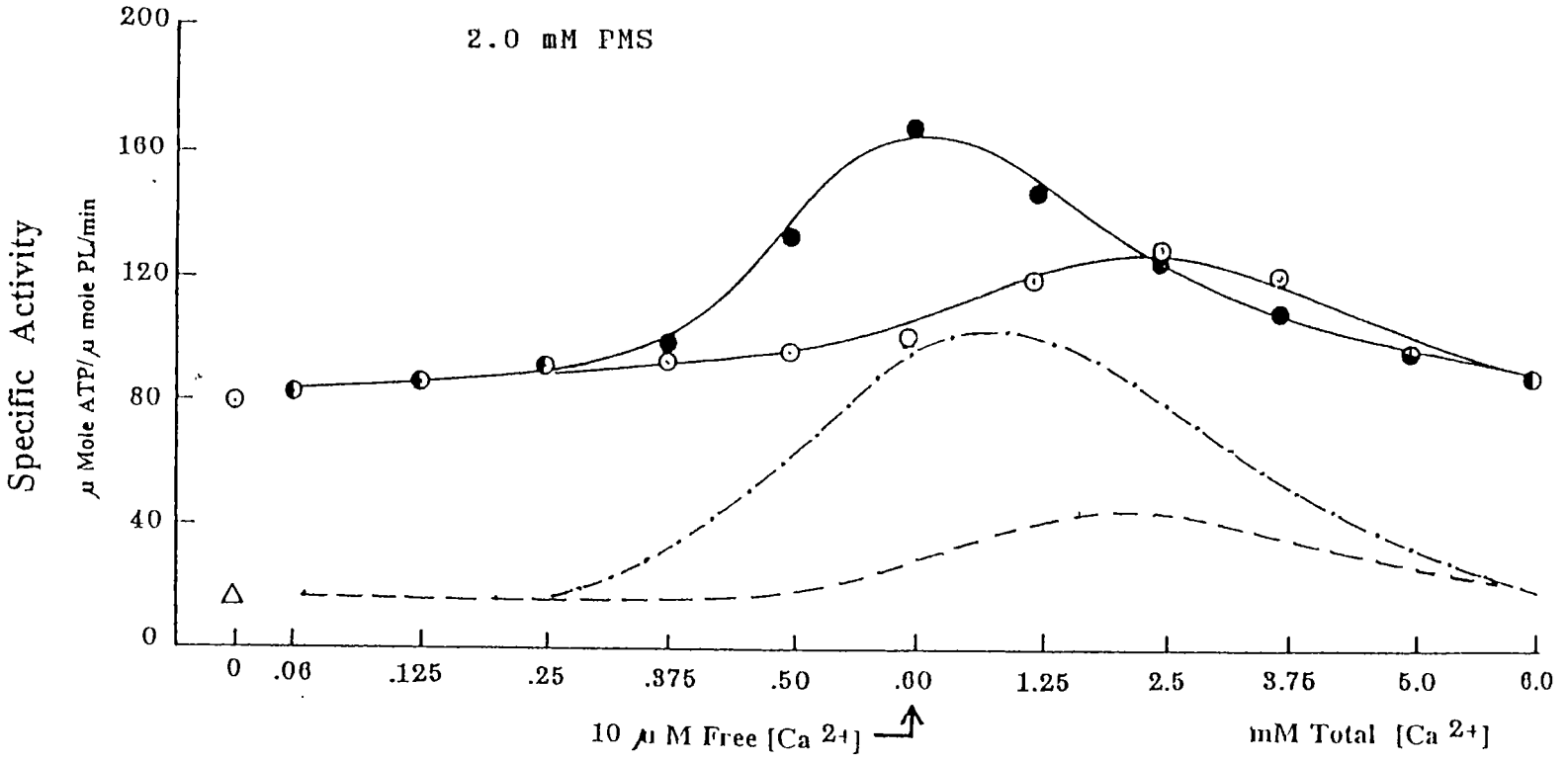


Figure 49: The responses of Ca^{2+}/Mg^{2+} -ATPase from oxidised erythrocyte membranes to changes in Ca^{2+} ions concentration. Intact normal human erythrocytes were treated with 2.0 mM phenazine methosulfate at 37 °C, 1 hr, and membranes were prepared as described in "Materials and Methods". The enzyme activity was assayed by coupled enzyme method with, -o-, and without, -o-, addition of calmodulin; normal controls with,.-., and without,---, addition of calmodulin.

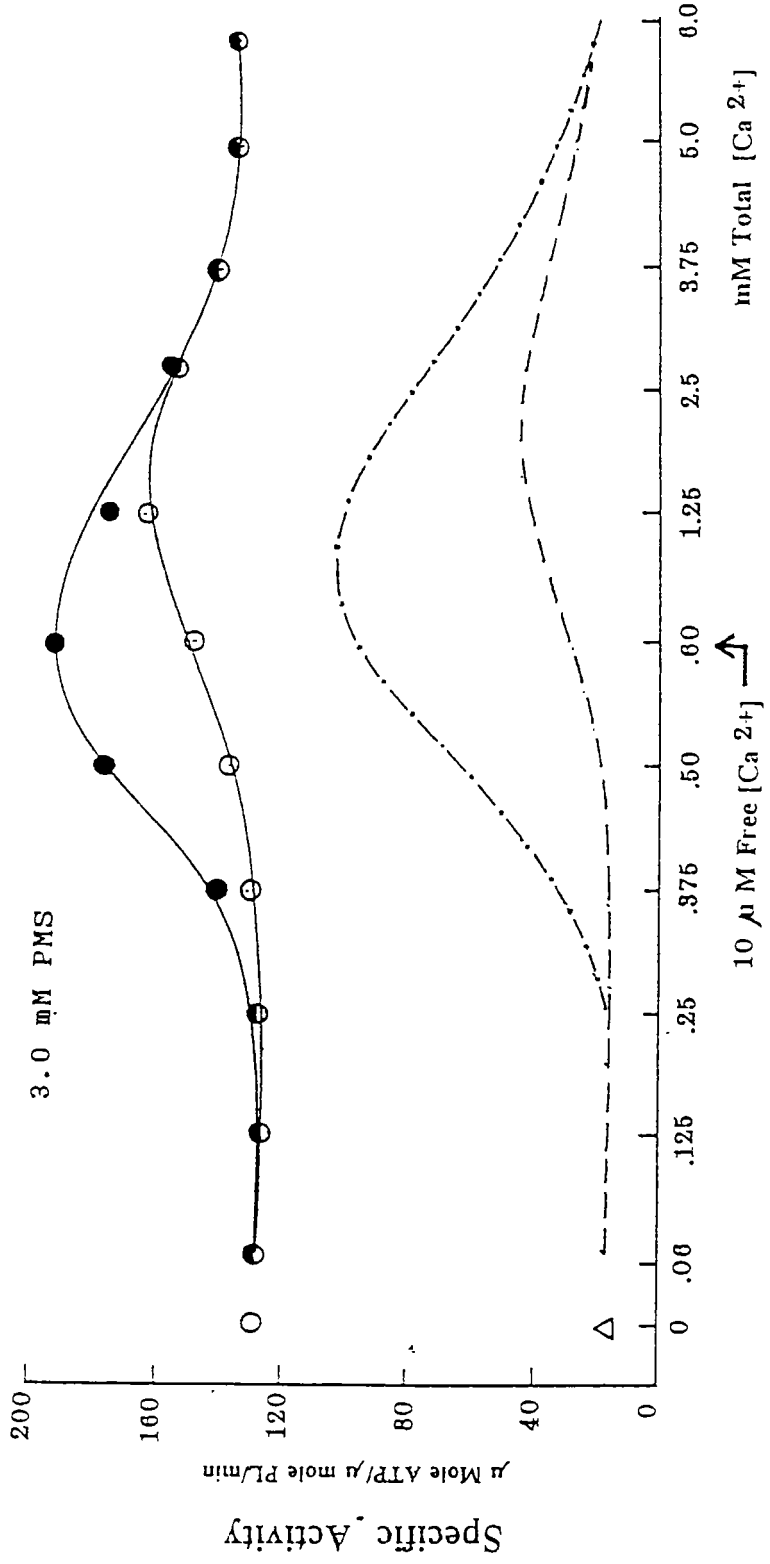


Figure 50: The responses of Ca^{2+}/Mg^{2+} -ATPase from oxidised erythrocyte membranes to changes in Ca^{2+} ions concentration. Intact normal human erythrocytes were treated with 3.0 mM phenazine methosulfate at 37 °C, 1 hr, and membranes were prepared as described in "Materials and Methods". The enzyme activity was assayed by coupled enzyme method with, -o-, and without, -o-, addition of calmodulin; normal controls with,.-.., and without,---, addition of calmodulin.

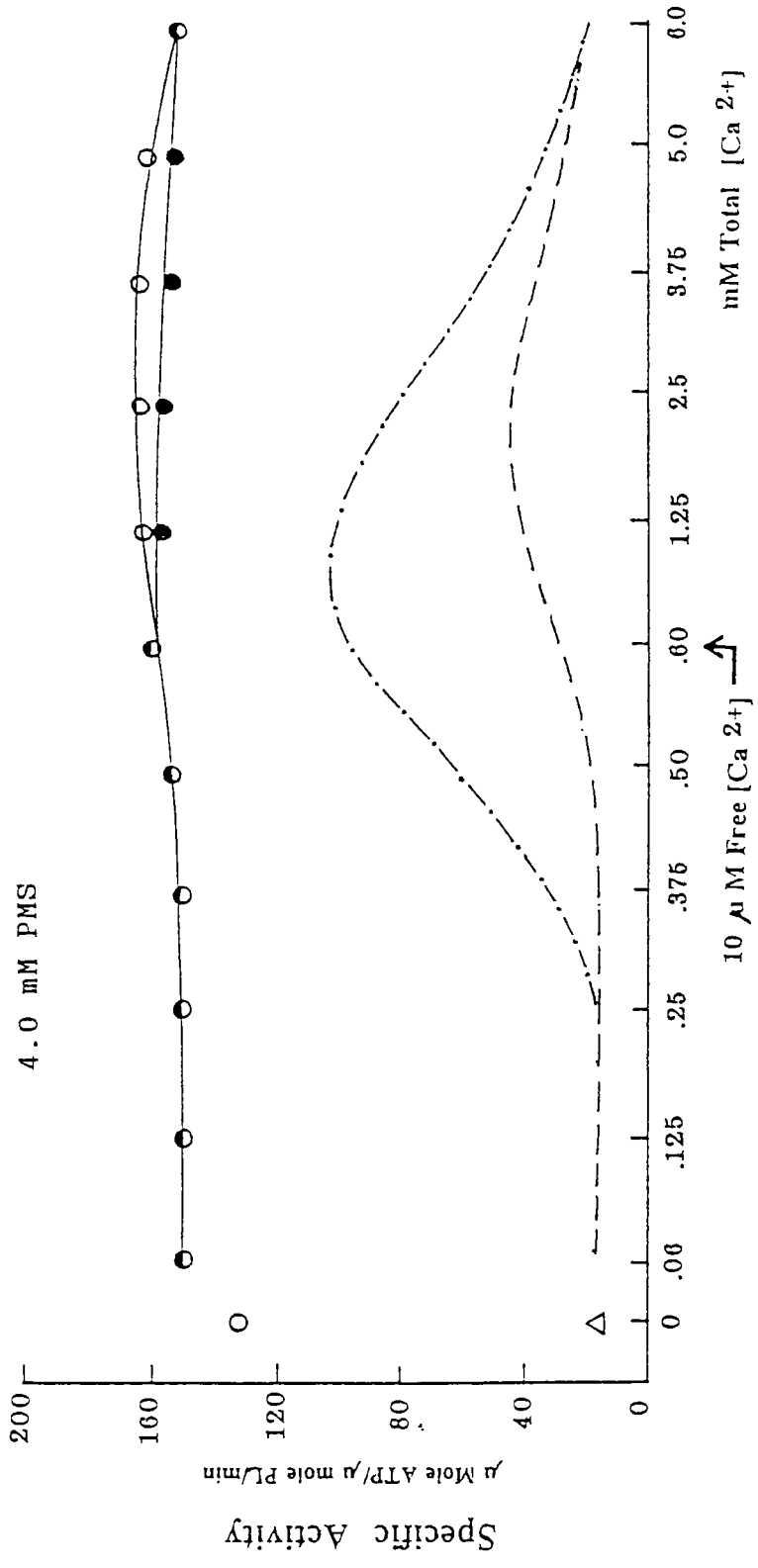


Figure 51: The responses of Ca^{2+}/Mg^{2+} -ATPase from oxidised erythrocyte membranes to changes in Ca^{2+} ions concentration. Intact normal human erythrocytes were treated with 4.0 mM phenazine methosulfate at 37 °C, 1 hr, and membranes were prepared as described in "Materials and Methods". The enzyme activity was assayed by coupled enzyme method with, -o-, and without, -o-, addition of calmodulin; normal controls with,.-.-, and without,---, addition of calmodulin.

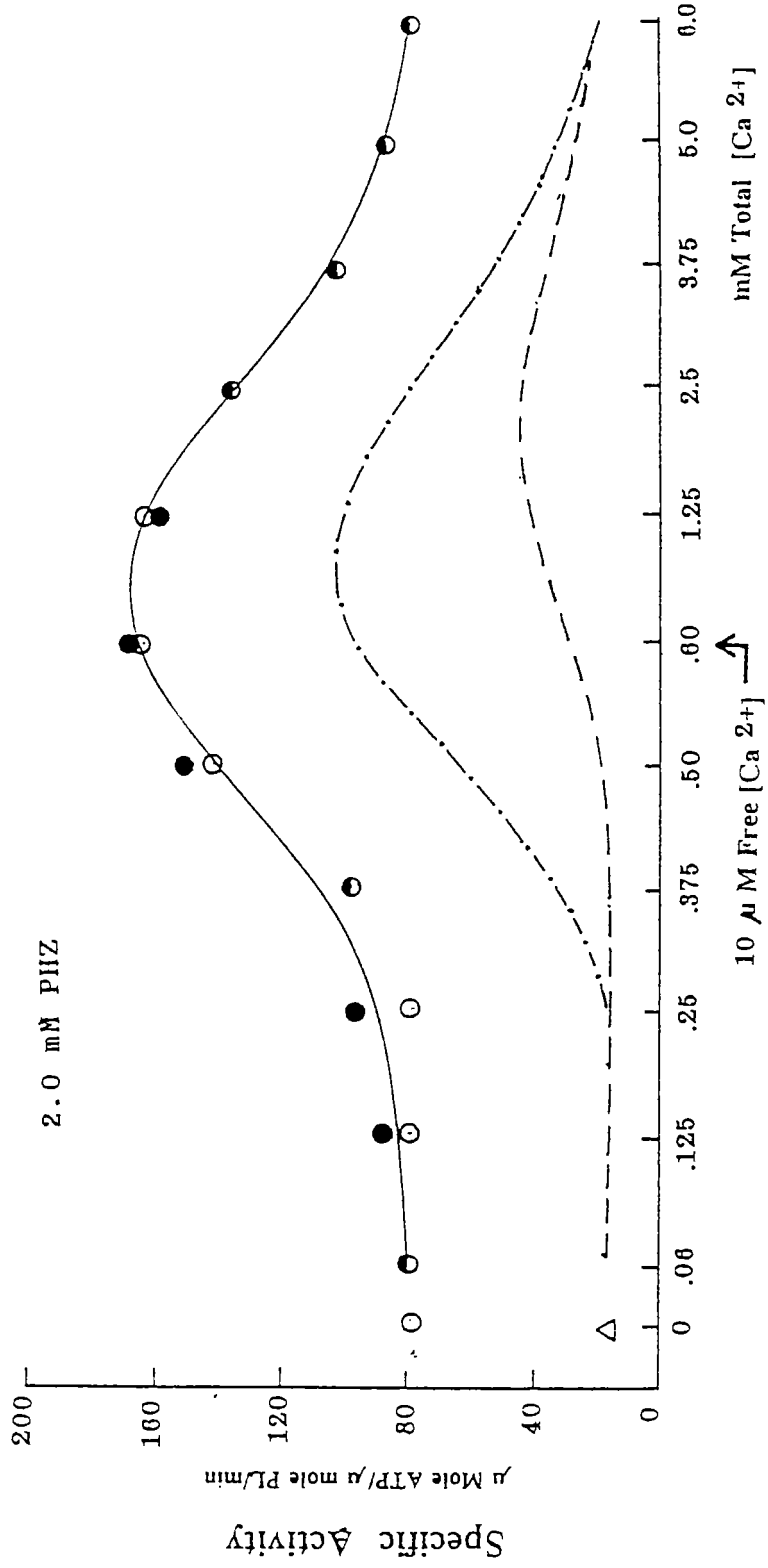


Figure 52: The responses of Ca^{2+}/Mg^{2+} -ATPase from oxidised erythrocyte membranes to changes in Ca^{2+} ions concentration. Intact normal human erythrocytes were treated with 2.0 mM phenylhydrazine at 37 °C, 1 hr, and membranes were prepared as described in "Materials and Methods". The enzyme activity was assayed by coupled enzyme method with, -o-, and without, -o-, addition of calmodulin; normal controls with,.-.-, and without,---, addition of calmodulin.

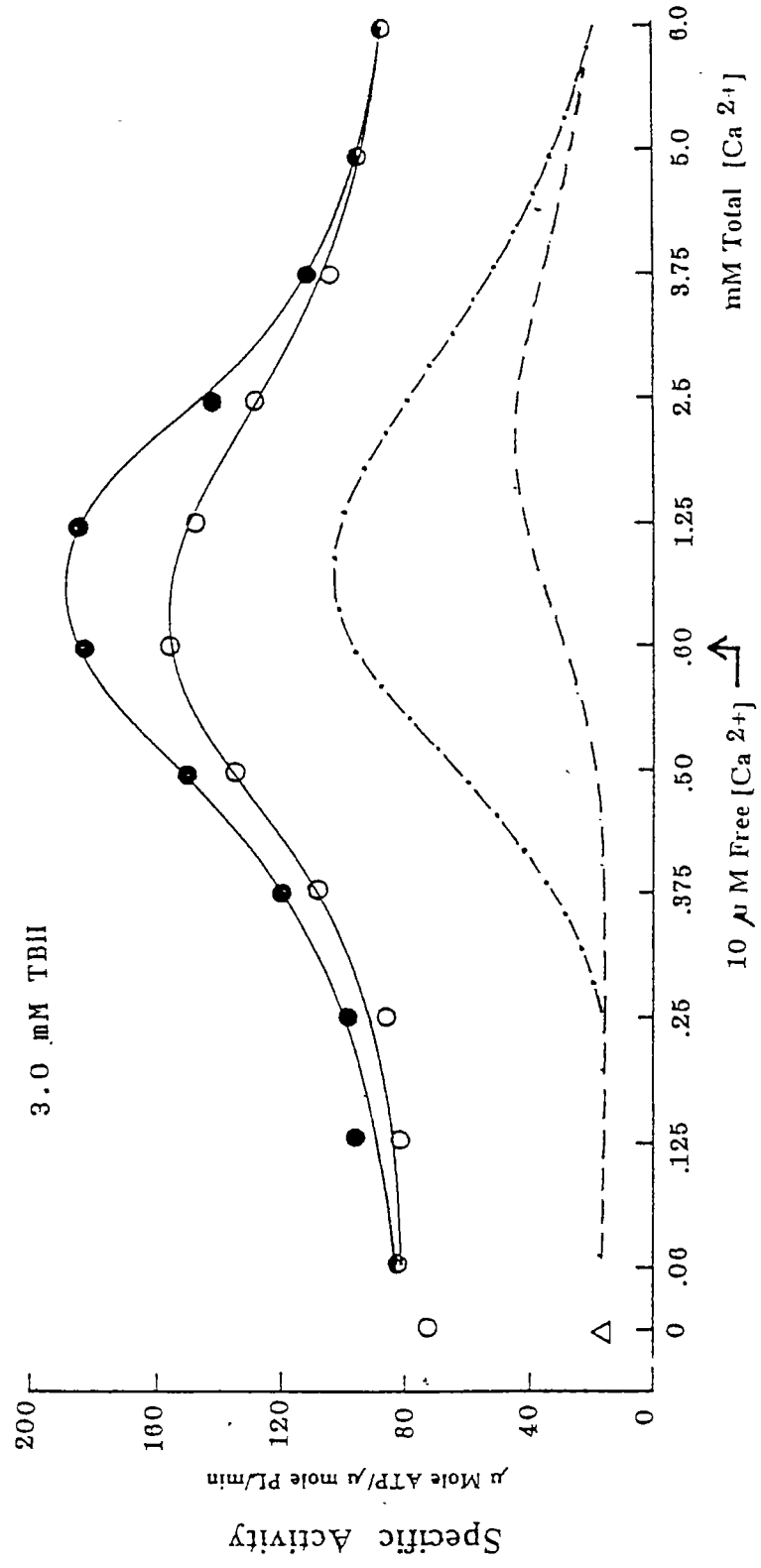


Figure 53: The responses of Ca^{2+}/Mg^{2+} -ATPase from oxidised erythrocyte membranes to changes in Ca^{2+} ions concentration. Intact normal human erythrocytes were treated with 3.0 mM tert-butylhydroperoxide at 37 °C, 1 hr, and membranes were prepared as described in "Materials and Methods". The enzyme activity was assayed by coupled enzyme method with, -o-, and without, -o-, addition of calmodulin; normal controls with,.-.-, and without,---, addition of calmodulin.

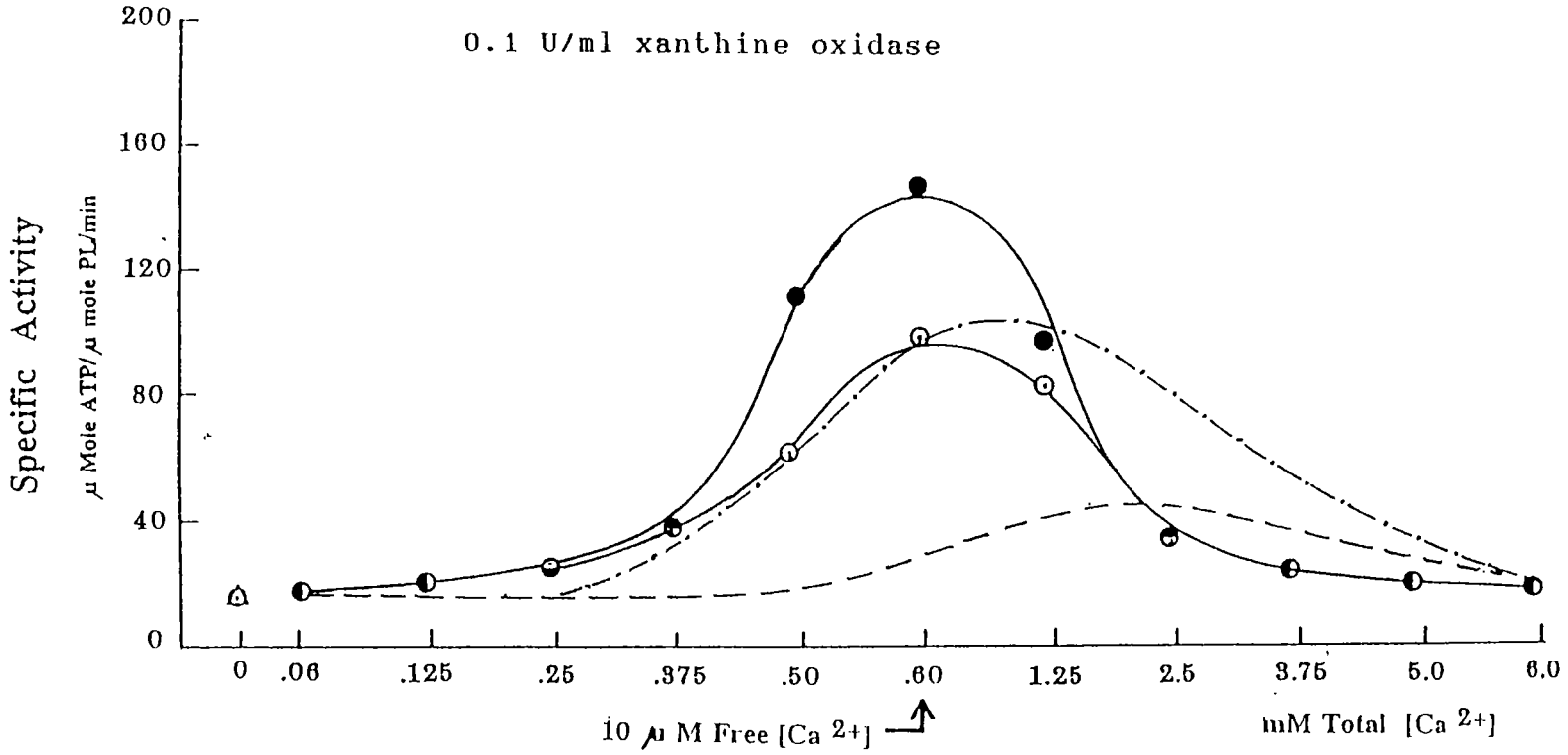


Figure 54: The responses of Ca^{2+}/Mg^{2+} -ATPase from oxidised erythrocyte membranes to changes in Ca^{2+} ions concentration. Normal human erythrocyte membranes were treated with 0.1 U/ml xanthine oxidase at 37 °C, 15 min, and membranes were prepared as described in "Materials and Methods". The enzyme activity was assayed by coupled enzyme method with, -o-, and without, -o-, addition of calmodulin; normal controls with,.-., and without,---, addition of calmodulin.

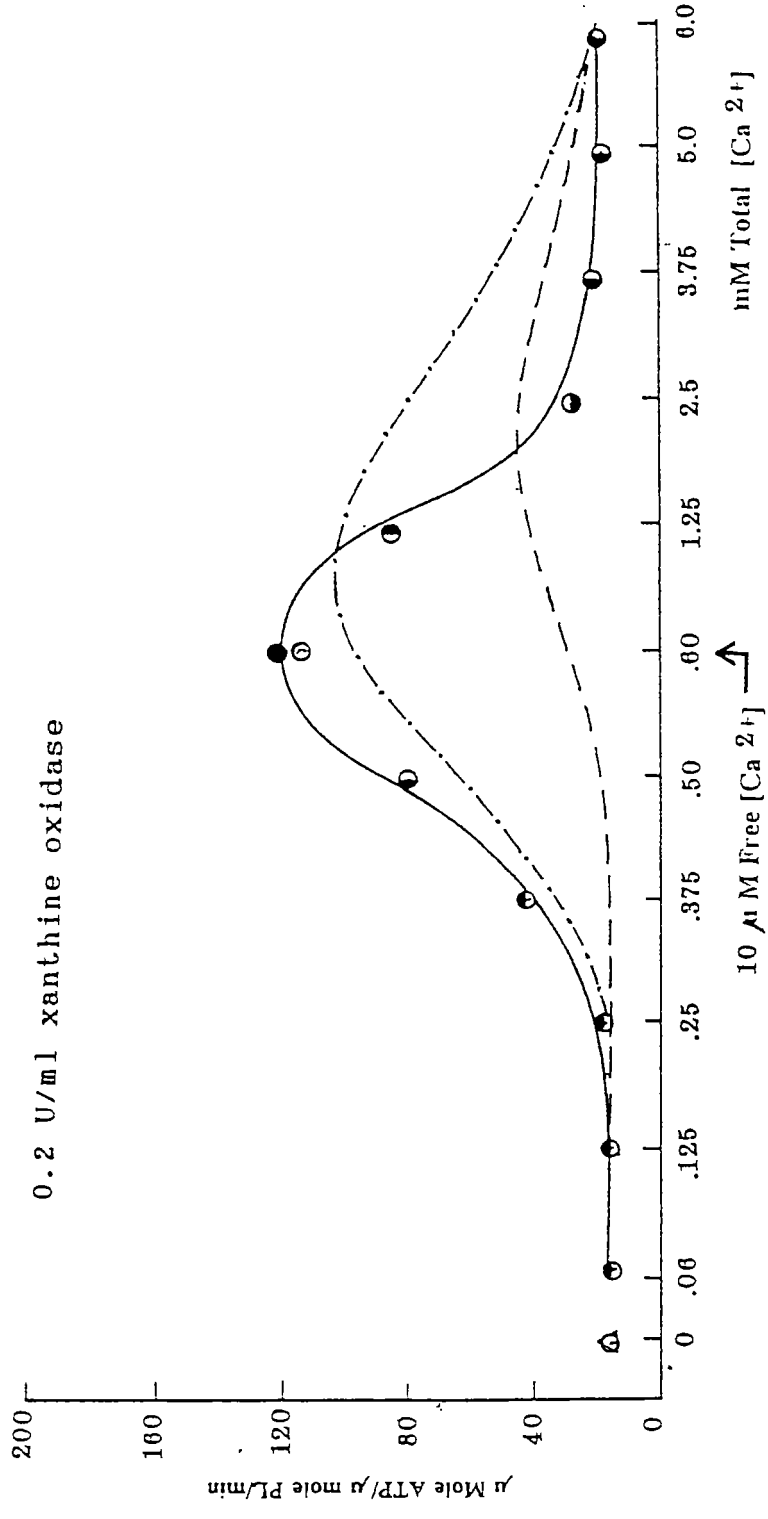


Figure 55: The responses of Ca^{2+}/Mg^{2+} -ATPase from oxidised erythrocyte membranes to changes in Ca^{2+} ions concentration. Normal human erythrocyte membranes were treated with 0.2 U/ml xanthine oxidase at 37 °C, 15 min, and membranes were prepared as described in "Materials and Methods". The enzyme activity was assayed by coupled enzyme method with, -o-, and without, -o-, addition of calmodulin; normal controls with,.-.., and without,---, addition of calmodulin.

ATPase activity. Figures 34, 36 and 38 show that the effects of the oxidants on activation of the Ca^{2+} / calmodulin-independent ATPase activity were concentration dependent. The results also supported the suggestion that the total measurable Ca^{2+}/Mg^{2+}-ATPase activities were due to the Ca^{2+}/calmodulin-independent ATPase activity. The results suggested further that activation of the Ca^{2+}/calmodulin-independent ATPase activity as ocurring in thalassemia samples may be caused by the presence of oxygen free- radicals generated in the red blood cells.

The question then arose whether the alterations in the erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase were the consequnces of the stimulation of the cytosolic Ca^{2+}-dependent protease(s). Normal human erythrocyte membrane preparations were oxidised with either phenazine methosulfate or xanthine oxidase in absence of Ca^{2+} ions in the buffer. Then ATPase activities were measured by the coupled enzyme medium under 3 conditions: i.e. in absence of both Ca^{2+} ions and bovine brain calmodulin, in the presence of Ca^{2+} ions but absence of calmodulin, and in the presence of both Ca^{2+} ions and calmodulin. The results are shown in Figure 56 and 57.

The effects of phenazine methosulfate (PMS) on the cell-free membrane preparations (Figure 56) were very similar to those observed in intact cell experiments (Figure 34). It was observed that the oxidants PMS (Figure 34 and 56), TBH (Figure 36), and PHZ (Figure 38) caused decrease in the Ca^{2+}/calmodulin-

dependent ATPase activity at low concentrations. At higher concentrations of the oxidants, the activity was due to that of calmodulin-unstimulated Ca^{2+} -ATPase and/or the Ca^{2+}/ calmodulin-independent ATPase. Moreover, the calmodulin-stimulating response of the enzyme was completely inhibited. Xanthine oxidase which has been known to generate peroxide radicals also exerted similar effects on the erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase, though the effect on activation of the Ca^{2+}/calmodulin-independent ATPase activity were not as high as that observed from PMS, TBH, or PHZ oxidation (Figure 57).

These experiments supported the idea that the calmodulin-dependent Ca^{2+}/Mg^{2+}-ATPase activity was inactivated in thalassemic erythrocytes whist the Ca^{2+}/calmodulin-independent ATPase activity was stimulated. This would reflect the decoupling of Ca^{2+}-translocation from the ATPase activity of the enzyme. The effects were caused by the presence of oxygen free-radicals in thalassemic red blood cells.

9. Ability of the Thalassemic Erythrocyte Membrane Ca^{2+}/Mg^{2+} -ATPase to Form the Phosphoenzyme Intermediate

In order to elucidate the dysfunction of the Ca^{2+}-pump in thalassemic erythrocytes, the ability of the Ca^{2+}/Mg^{2+}-ATPase to form phosphoenzyme intermediate was examined. Formation of the phosphoenzyme is enhanced by incubation of the erythrocyte membrane with γ-^{32}P-ATP in

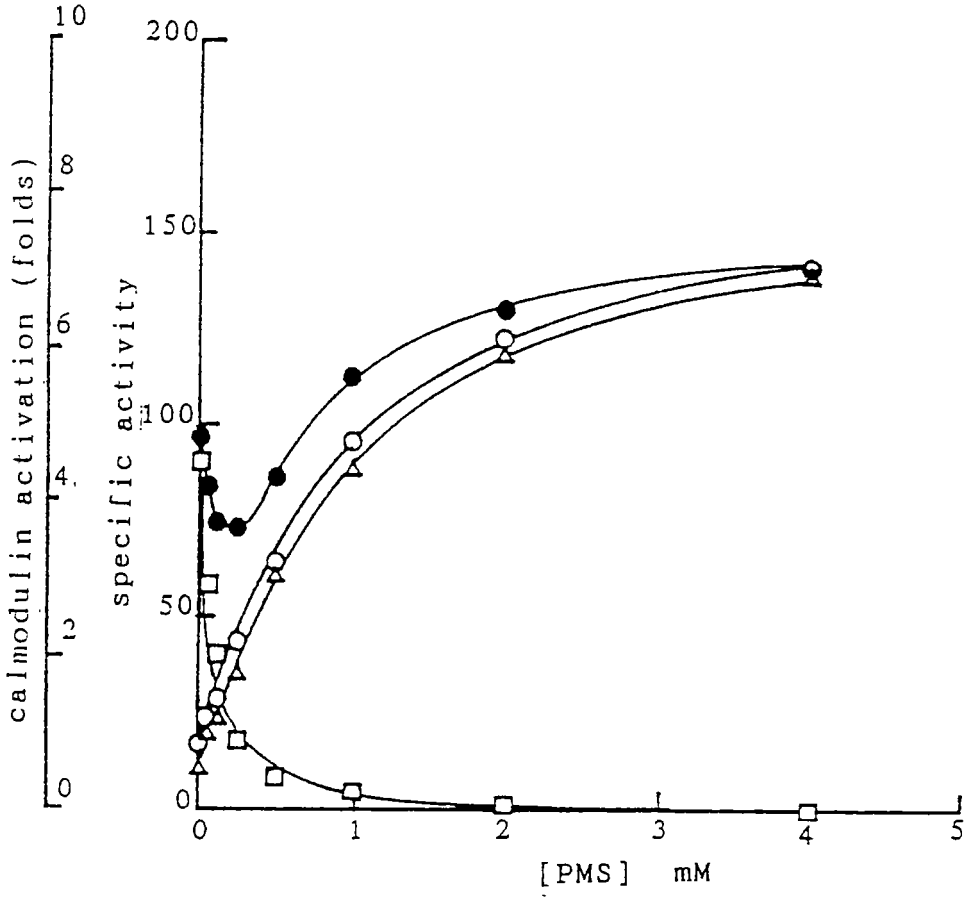


Figure 56: Effects of phenazine methosulfate (PMS) on erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase. Normal human erythrocyte membrane preparations were oxidised at 37 °C, 15 min., and enzyme activity was assayed by coupled enzyme method as described in "Materials and Methods". The assay conditions were: Ca^{2+} present but with,-o-,or without,-o-, addition of calmodulin; -v-, absent of both Ca^{2+} ions and calmodulin; -□-, calmodulin activation.

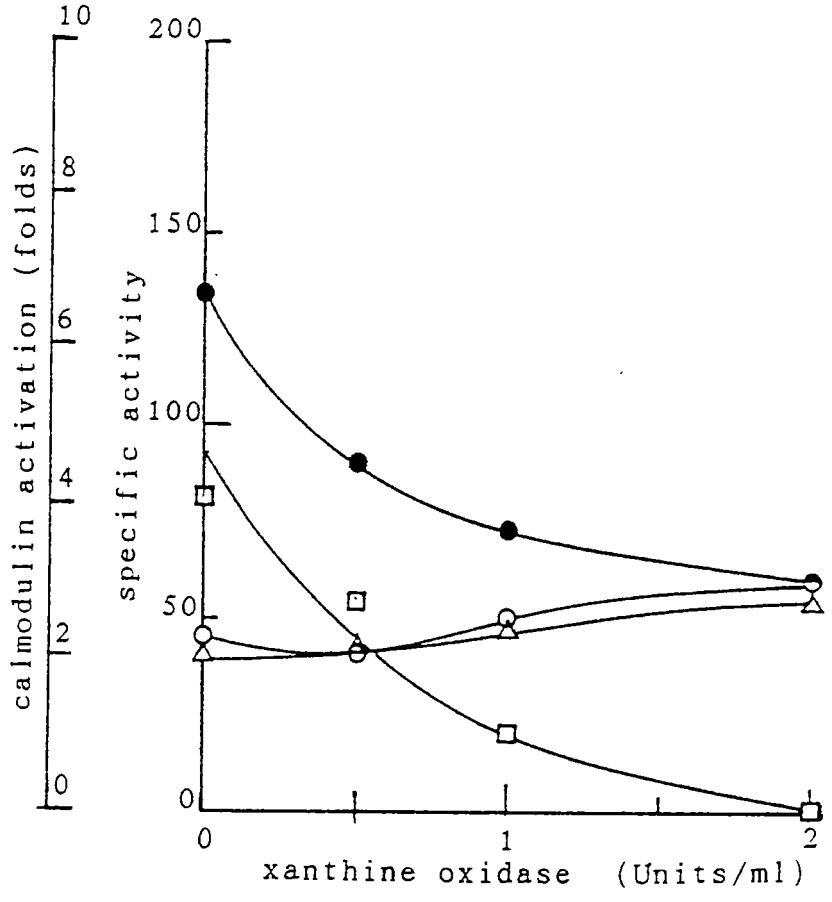


Figure 57: Effects of xanthine oxidase on erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase. Normal human erythrocyte membrane preparations were oxidised at 37 °C, 15 min., and enzyme activity was assayed by coupled enzyme method as described in "Materials and Methods". The assay conditions were: Ca^{2+} present but with,-o-,or without,-o-, addition of calmodulin; -v-, absent of both Ca^{2+} ions and calmodulin; -[]-, calmodulin activation.

the presence of Ca^{2+} and La^{3+} ions. The membrane proteins were analysed by acidic SDS-PAGE. The presence of the phosphoenzyme was visualized on X-rays film by autoradiography.

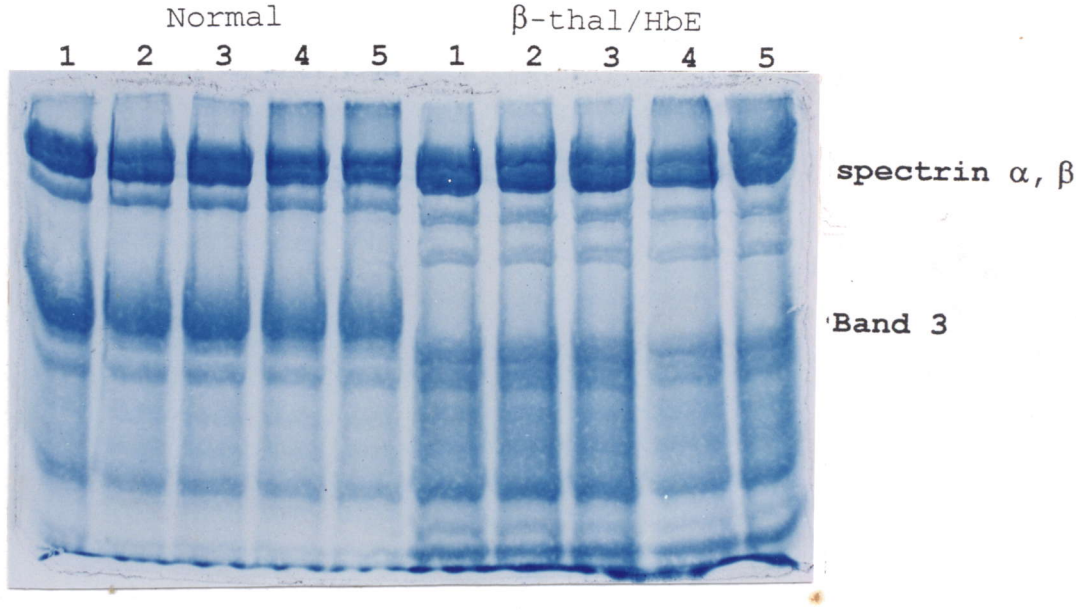
The results in Figure 58 showed the enhancing effect of La^{3+} ions on the formation of phosphoenzyme in membranes from normal control and β-thalassemia/HbE disease. The Ca^{2+}-pump appeared in between ankyrin and Band 3 protein in the acidic SDS-PAGE. Spectrin (β-form) was also phosphorylated. However, phosphorylation of the spectrin was independent of Ca^{2+} ions but was inhibited by La^{3+} ions. In the medium containing both Ca^{2+} and La^{3+} ions, another faster-moving ^{32}P-labelled band appeared on the autoradiograph at the same position of Band 3 protein. This band would represent the truncated form of the pump.

Figures 59 and 60 showed the results of phosphoenzyme formation of the Ca^{2+}-pump from erythrocytes of HbH disease, HbH/HbCS, homozygous HbCS, homozygous β-thalassemia, nonsplenectomized β-thalassemia/HbE, and splenectomized β-thalassemia/HbE. Each lane on acidic SDS-PAGE and autoradiograph represented sample from a different individual. It was obviously seen that the Ca^{2+}-pump from all cases lost the ability to form the phosphoenzyme.

The results from SDS-PAGE analysis also indicated that the membrane skeletal proteins of erythrocytes from all types of thalassemia were degraded to a certain extent.

Figure 58: Effects of Ca^{2+} and La^{3+} on the erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase in the formation of phosphoenzyme. Normal and thalassemic erythrocyte membranes were incubated under indicating conditions with γ-^{32}P-ATP at 25 °C, 20 seconds, as described in "Materials and Methods": 1; EGTA, 2; Ca^{2+}, 3; Ca^{2+} and La^{3+}, 4; Hydroxylamine, 5; Ammonium acetate.
a. Acidic SDS-PAGE ; b. Autoradiography

a. Acidic SDS-PAGE



b. Autoradiography

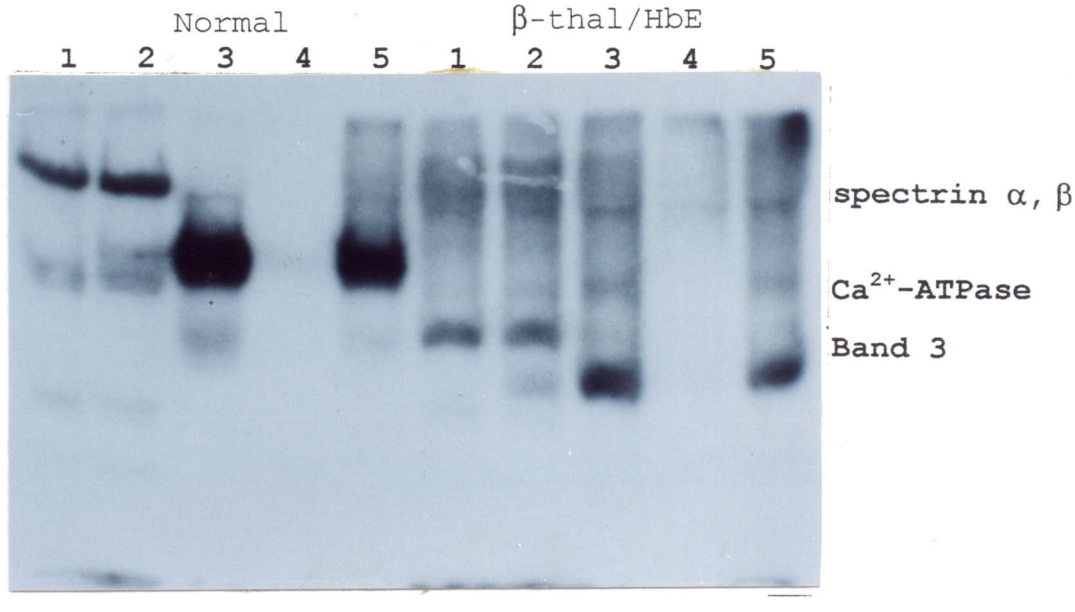
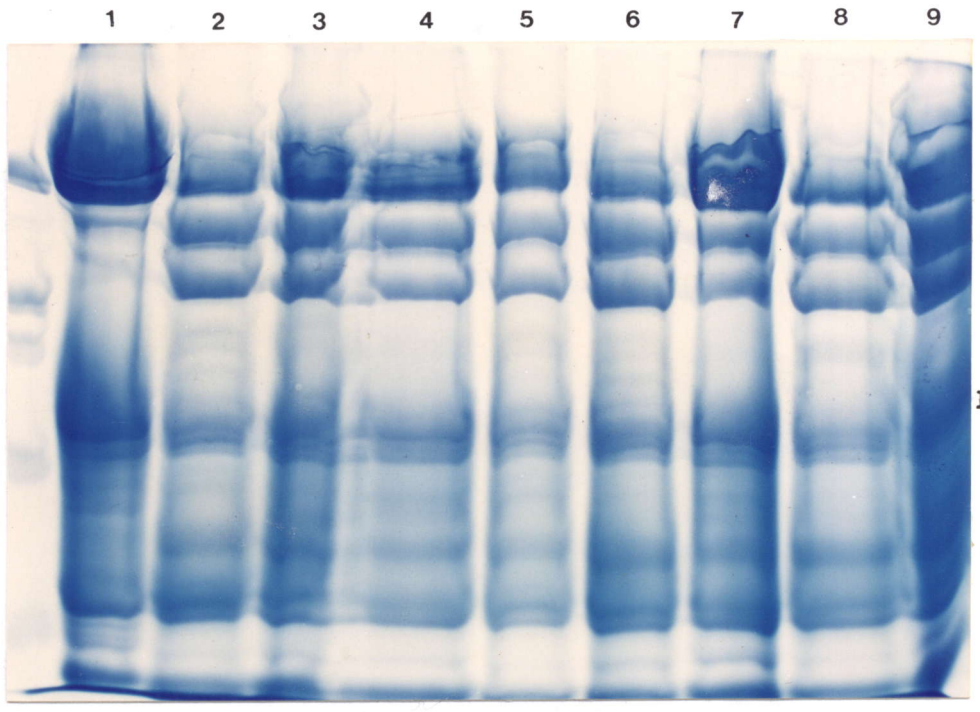


Figure 59: Formation of phosphoenzyme in the Ca^{2+}/Mg^{2+}-ATPase from thalassemic erythrocyte membranes.I. The membranes were incubated with γ-^{32}P-ATP in the presence of Ca^{2+} and La^{3+} at 25 °C, 20 seconds, as describes in "Materials and Methods", prior to analysis by acidic SDS-PAGE (a), and autoradiography (b).

1, normal control; 2, homozygous β-thalassemia; 3, 9, HbH;

4, 7, splenectomized β-thal/HbE; 5, HbH/HbCS; 6, homozygous β-thalassemia; 8, HbCS.

a. Acidic SDS-PAGE



b. Autoradiography

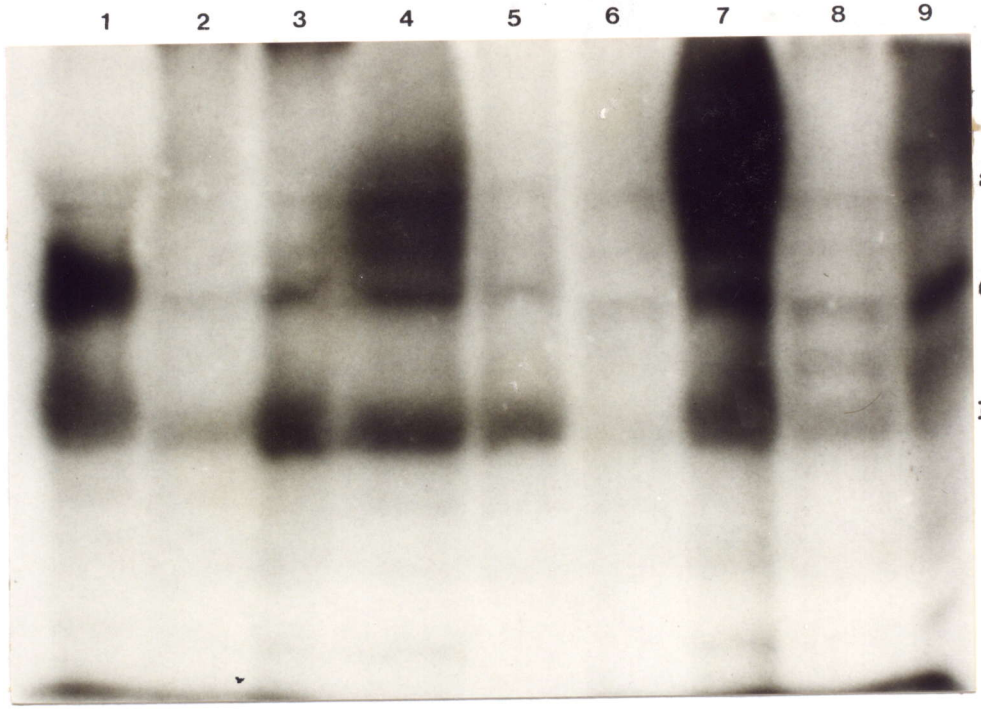
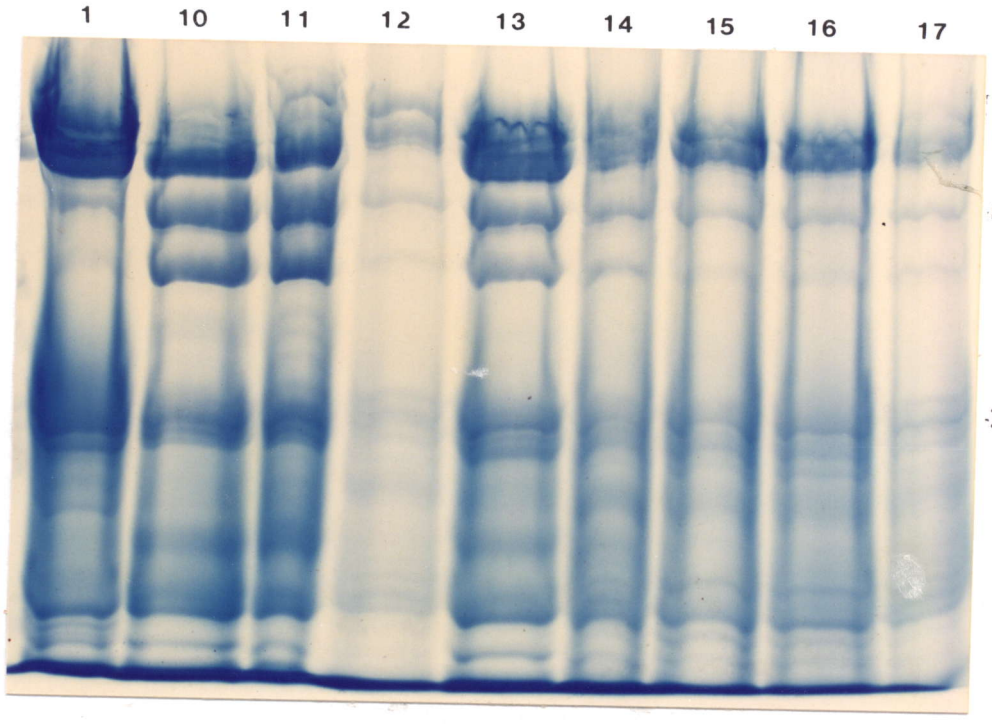
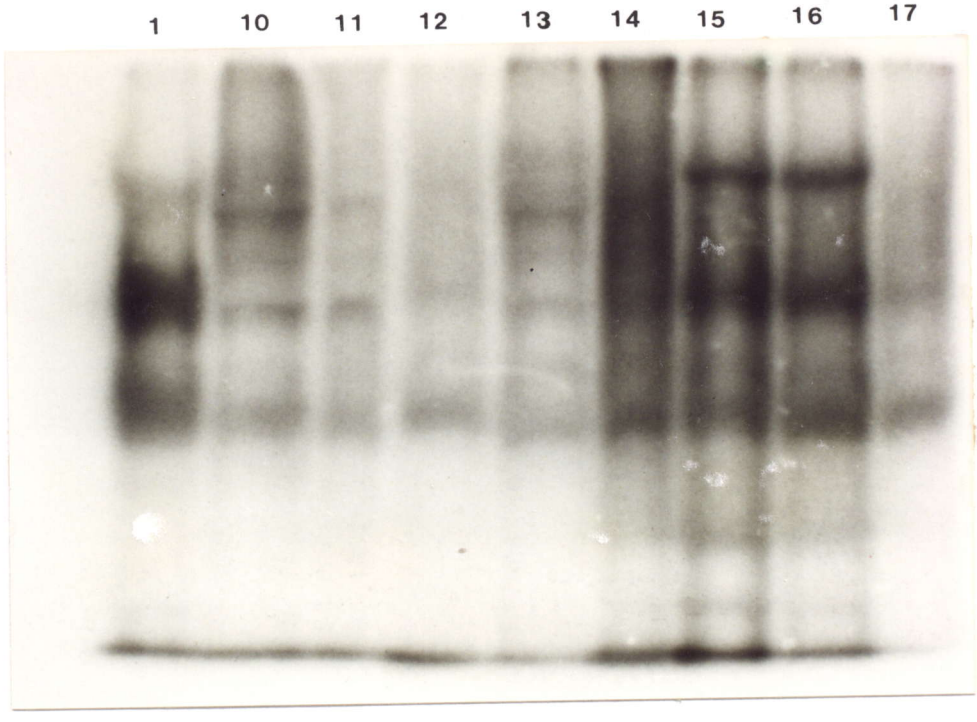


Figure 60: Formation of phosphoenzyme in the Ca^{2+}/Mg^{2+}-ATPase from thalassemic erythrocyte membranes.II. The membranes were incubated with γ-^{32}P-ATP in the presence of Ca^{2+} and La^{3+} at 25 °C, 20 seconds, as describes in "Materials and Methods", prior to analysis by acidic SDS-PAGE (a), and autoradiography (b).
1, normal control; 10, 13, β-thal/HbE; 11, HbH; 12, 14, 15, 16, 17, splenectomized β-thal/HbE.

a. Acidic SDS-PAGE



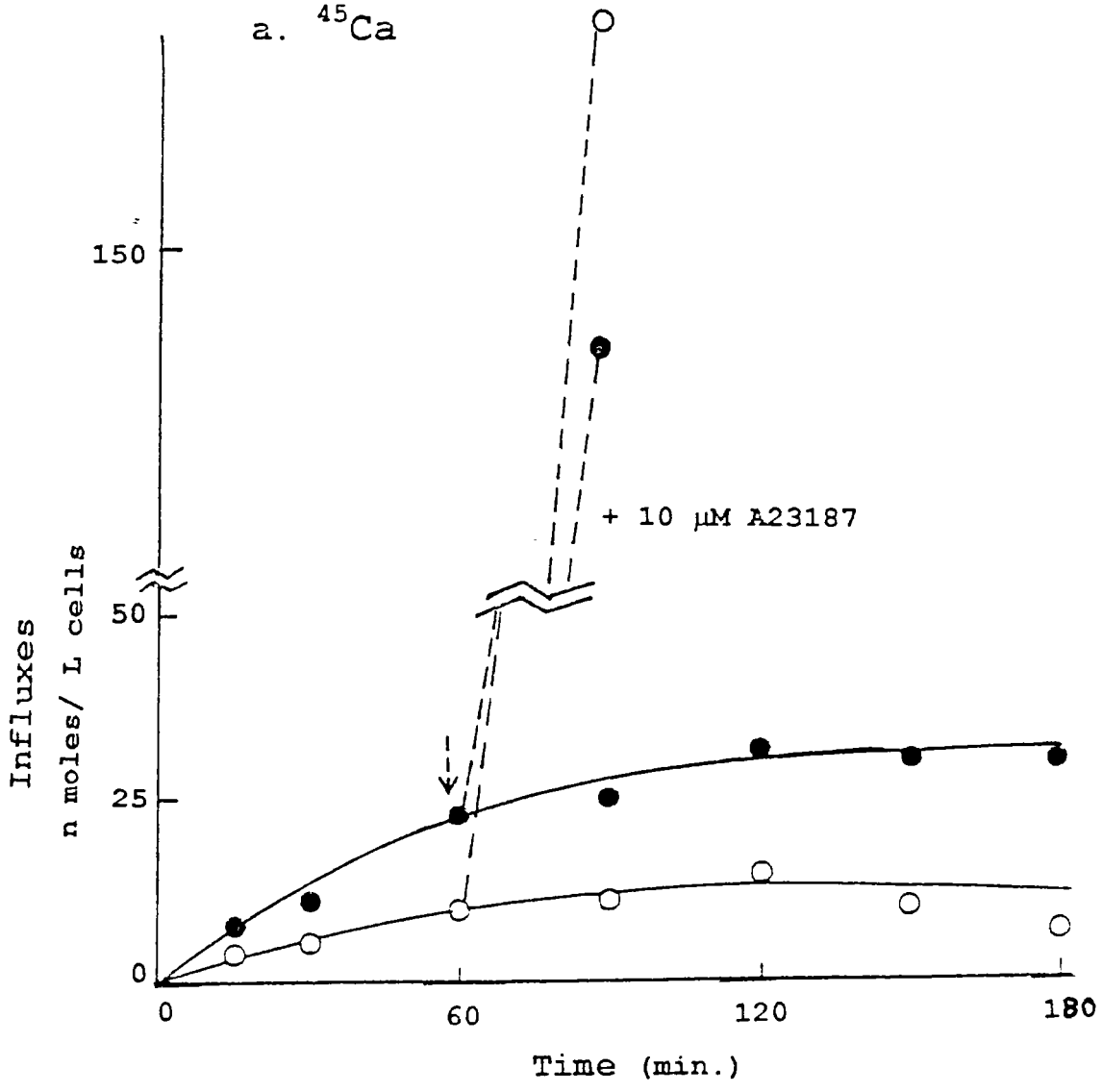
b. Autoradiography



10. Determination of the Effects of Oxygen Free-Radicals on Erythrocyte Membrane Permeability

The effects of oxygen free-radicals on perturbation of erythrocyte calcium homeostasis by alteration of the permeability of the membrane were also investigated. Normal human erythrocytes were treated to deplete ATP in order to inhibit the Ca^{2+} -pump activity. The cells were oxidised by phenazine methosulfate. Then, the permeability of the membrane to ^{45}Ca ions was compared to that of ^{3}H-L-glucose. Labelling experiments were performed in isotonic NaCl buffer and isotonic KCl buffer. The results are shown in Figure 61 and 62. The results obtained from experiments in both buffer were similar. Influx of ^{3}H-L-glucose into the oxidised erythrocytes was lower than than normal control. This indicated that the simple diffusion permeability of the erythrocyte membrane was reduced upon oxidation by oxygen free-radicals. In contrast, influx of ^{45}Ca ions into the oxidised erythrocytes was increased about 2.5 fold. The increase ^{45}Ca influx was not due to the oxidant-induced leakage of the erythrocyte membrane because large ^{45}Ca influx was observed after addition of Ca^{2+} ionophore, A23187. The results implied that the transport of Ca^{2+} ions into the erythrocyte was not by simple diffusion, and this specific mechanism was stimulated by oxygen free-radicals.

Figure 61: Effects of oxygen free-radicals on the permeability of the erythrocyte membranes.I. Normal human erythrocytes were treated to ATP-depletion and oxidised by 2mM PMS in isotonic KCL buffer. Influxes of ^{45}Ca and ^{3}H-L-glucose were observed in isotonic NaCl buffer: -o-, control; -o-, oxidised.


a. ^{45}Ca
150
50
25
0
+ 10 μM A23187
Influxes
n moles/ L cells
0
60
120
180
Time (min.)

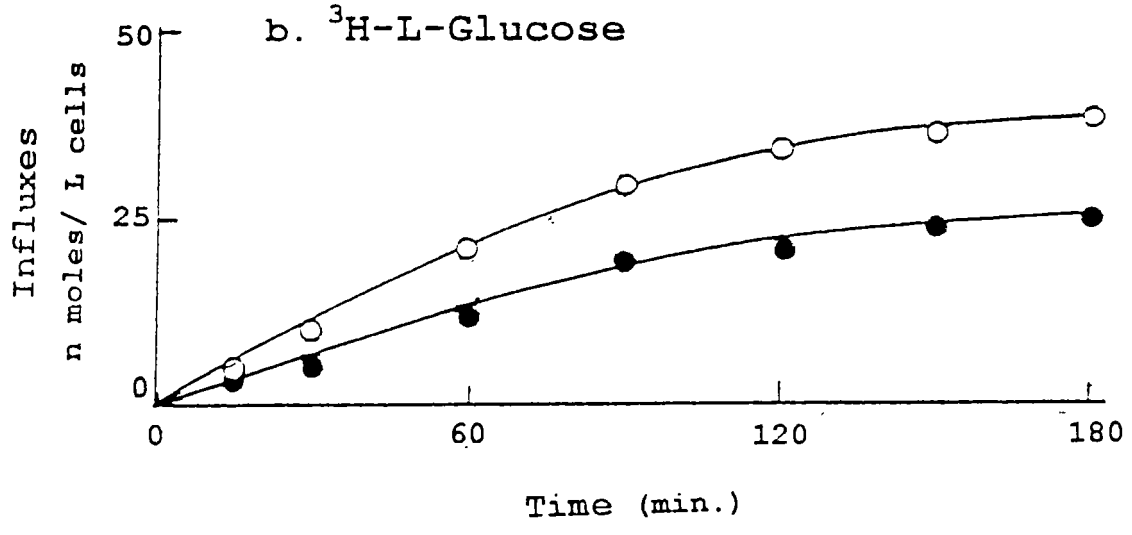
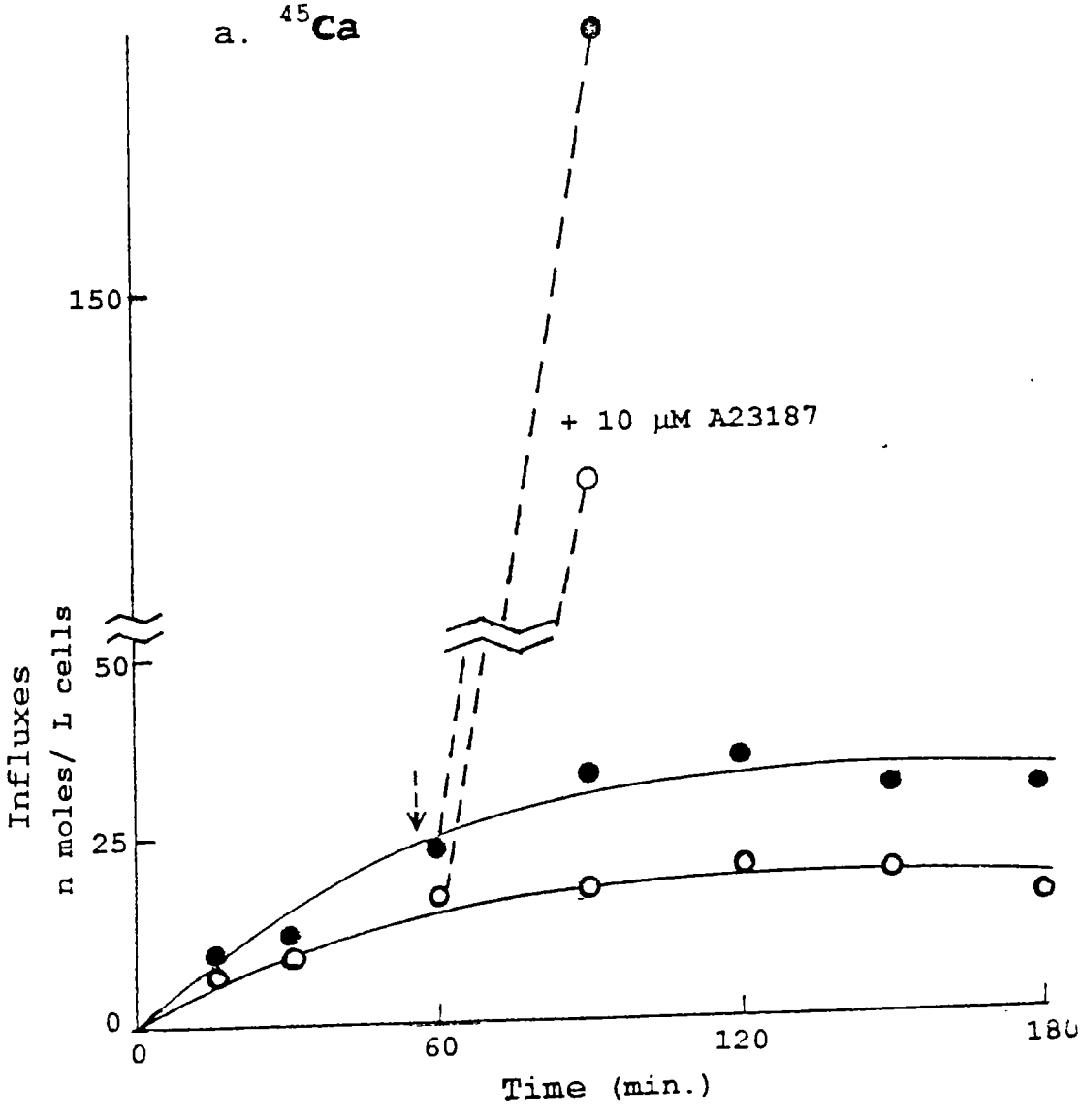
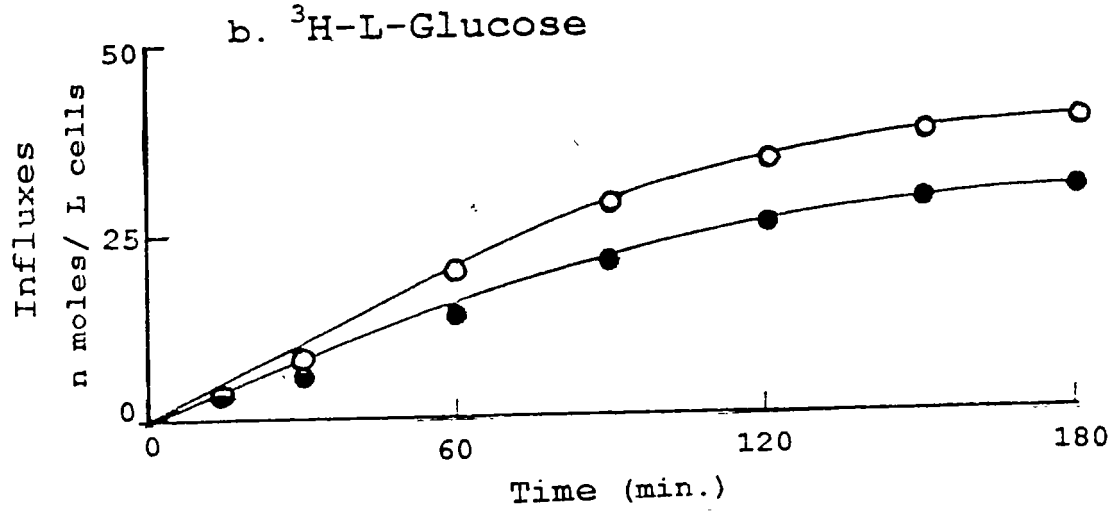

b. ^{3}H-L-Glucose
50
25
0
Influxes
n moles/ L cells
0
60
120
180
Time (min.)

Figure 62: Effects of oxygen-free radicals on the permeability of the erythrocyte membranes.II. Normal human erythrocytes were treated to ATP-depletion and oxidised by 2 mM PMS in isotonic KCL buffer. Influxes of ^{45}Ca and ^{3}H-L-glucose were observed in isotonic KCl buffer: -o-, control; -o-, oxidised.


a. 45Ca
+ 10 μM A23187
Influxes
n moles/ L cells
150
50
25
0
0
60
120
180
Time (min.)


b. 3H-L-Glucose
Influxes
n moles/ L cells
50
25
0
0
60
120
180
Time (min.)

CHAPTER 4

DISCUSSION

It has been generally accepted that thalassemic erythrocyte membranes contain an increased amount of bound-hemoglobin in oxidised form called "hemichrome" (76). The oxidised hemoglobin is usually associated with a cytoskeletal protein, the Band 3 protein. Both α- and β- globin have been found to be increased on the membrane, but only the α-chains are bound to the cytoskeletons (152). The interaction between the oxidised hemoglobin and the cytoskeletons is likely to be hydrophobic (153, 154). However, the interaction is so strong that it can be disrupted only in very high percentage of sodium dodecylsulfate (SDS) (155). Similar observations have been found in membrane preparations from intact normal erythrocytes exposed to oxidant-generating agents such as phenylhydrazine (156). In this study, increased amount of methemoglobin was also found in membrane preparations from thalassemic erythrocytes and erythrocytes exposed to phenylhydrazine, phenazine methosulfate, and tert-butylhydroperoxide. The contaminating hemoglobin thus interfered with the calculation of the specific activity of the enzyme Ca^{2+}/Mg^{2+}-ATPase when expressed as μmole ATP hydrolysed/minute/mg membrane protein. To overcome this problem, the specific activity of the enzyme was expressed, in this study, as μmole ATP hydrolysed/ μmole membrane phospholipid/ minute.

Increased calcium concentration have been reported in erythrocytes with β-thalassemia intermedia

from the Middle East (114, 115, 116) and β-thalassemia/ hemoglobin E which is prevalent in Thailand (174). Loss of cellular deformability (47), alterations in the function of cytoskeletal proteins (76), and perturbations to the membrane phospholipid asymmetry (65, 66, 67) have been observed in thalassemia samples. The deleterious effects of cellular calcium overload on cytoskeletal degradation(85, 86), alterations of membrane phospholipid asymmetry (87, 88), decreased deformability (89), and membrane vesiculation (90, 91) have also been reported. Hence, it was important to investigate the mechanism of calcium accumulation in the thalassemic erythrocytes.

Accumulation of calcium have been reported in erythrocytes other than thalassemia, e.g. G-6-P DH deficiency (118, 119), sickle cell disease (120, 121), and homozygous hemoglobin C disease (114). Abnormal function of Ca^{2+}/Mg^{2+}-ATPase (118, 123, 130, 131, 132, 133, 134, 159), alterations in the membrane permeability (159, 160), and changes in the calcium-binding properties of the membranes (161) have been suggested to be responsible for calcium accumulation in these abnormal erythrocytes.

The mechanism of calcium accumulation in thalassemic erythrocytes is not known. Since 1984, there has been only one report on the activity of Ca^{2+}/Mg^{2+}-ATPase in β-thalassemia intermedia erythrocytes. Shalev and coworkers (115) reported that the specific activities of the enzyme were not significantly

different among erythrocytes from non-splenectomized, splenectomized β-thalassemia intermedia and normal control. They suggested that an increased Ca^{2+} content in thalassemic erythrocytes was associated with the membrane defect. Increased Ca^{2+}-permeability in thalassemic erythrocytes has been reported by Wiley (117). However, Rhoda et al (114) and Bookchin et al (116) argued that Ca^{2+}-permeability was normal in erythrocytes from β-thalassemia intermedia and that the loaded calcium in these erythrocytes was associated with the abnormal compartmentation of the ions in endocytic inside-out vesicles.

In sickle cell disease and favic G-6-P DH deficiency, defective activity of Ca^{2+}/Mg^{2+}-ATPase was associated with the oxidative stress condition in these red blood cells (128, 130, 131). The conditions of oxidative stress, viz. the increase of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase, and the decrease of the antioxidant vitamin E and vitamin C content, have also been observed in α-thal 1/α-thal 2, α-thal 1/HbCS (175) and β-thal/HbE (176) erythrocytes. It is unlikely that the Ca^{2+}/Mg^{2+}-ATPase would escape from oxidative damage in the thalassemic erythrocytes. Hence, it was the purpose of this study that the oxidative inactivation of thalassemic erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase be investigated.

The results from the present study agreed with those from Shalev and coworkers (115) when the enzyme

activity was assayed at temperature 37-40 °C. However, such data seemed to be insufficient to indicate the status of the enzyme because multiple factors are required for its function, i.e. Ca^{2+} ions, calmodulin, membranes and coupling efficiency of the ATPase and the Ca2+ -affinity domains.

The detailed architecture of the plasma membrane Ca^{2+}/Mg^{2+}-ATPase has been reviewed extensively (94, 95, 96, 97, 98). The enzyme is a plasma membrane-bound polypeptide containing 2 active centers, i.e. the ATPase activity protruding into the cytoplasm, and the Ca^{2+}-translocation moiety locating at regions next to the transmembrane helices 4, 5, 6, 7, and 8. In absence of calmodulin, the calmodulin-binding domain, which is located on the proximal carboxyl terminal of the polypeptide, obstructs the Ca^{2+}-binding domain. Thus the Ca^{2+}-translocation is inhibited (148). The couple of the two activities has been described (160). The energy from the γ-phosphate moiety of ATP is transduced to the Ca^{2+}-translocation via phosphorylation at a specific aspartyl residue (residue number 475) (95, 103). It has been proposed that the phosphoenzyme intermediate changes the conformation and thus gates the Ca^{2+} ions to move from high-affinity domain (cytosolic surface) to low-affinity domain (outer surface).

The results from Arrhenius experiments (Figure 22) showed that, in absence of calmodulin activation, the profile of the enzyme activity was linear and the calculated activation energy was about 16 kcal. These

observations suggested that, in the absence of calmodulin, only one active center was functioning, i.e. the ATPase activity but not the Ca^{2+} translocation. Upon activation by calmodulin, the condition which the ATPase activity was coupled with the Ca^{2+} translocation, the ATPase activity was increased more than 3 folds and the Arrhenius profile showed a discontinuity at 32 ^{0}C with two different values of activation energy, 26 kcal below and 16 kcal above the break. When the activity of thalassemic Ca^{2+} /Mg^{2+} -ATPase was examined at temperature ranging from 14 - 40 ^{0}C, the differences among samples from normal control and thalassemia were obviously seen. The abnormalities of the enzyme in thalassemia samples could be demonstrated by loss of calmodulin stimulation, increased ATPase activity at all temperature assay, loss of the Arrhenius break, with a decreased activation energy at temperature below 32 ^{0}C. Elevation of the ATPase activity without calmodulin stimulation would direct a person to think that the enzyme was already activated, and it should be beneficial for the erythrocytes to pump out Ca^{2+} ions. However, experiments on controlled proteolysis by trypsin (Figure 29) indicated that removal of the calmodulin-binding domain from core enzyme, which is equivalent to the calmudulin activation (146, 161), could not alter the Arrhenius profiles of the enzyme activity. The truncated Ca^{2+} /Mg^{2+} -ATPase still showed an Arrhenius break at 32 ^{0}C, with unaltered values of activation energy.

The presence of the discontinuity in the Arrhenius plot have been reported in sarcoplasmic

reticulum Ca^{2+} ATPase. The observations were interpreted by differefent arguments. Measurements of rotational mobility of the protein component (177, 178) suggested an aggregation phenomenon. On the other hand, experiments employing hydrogen-deuterium exchange kinetics of the peptide NH protons (179, 180), saturation transfer electron spin resonance (181, 182), electron microscopic examination of freeze fractured vesicles (183), and chemical cross-linking in vitamin E-deficient dystrophic rabbit skeletal muscle (184) supported a conformational explanation. However, another explanation (146) suggested that the discontinuity would represent the characteristics of two parallel reactions, ATPase activity and Ca2+ translocation, carried out by two different active centers on one enzyme, with different values of activation energy. Whatever the cause of the discontinuity, this study indicates that thalassemic erythrocyte Ca^{2+}/Mg^{2+}-ATPase has lost its normal characteristics by some mechanism, following lipid peroxidation, resulting in uncoupling of the ATPase activity and the Ca2+ translocation.

The suggestion that the ATPase activity and the Ca2+ translocation were uncoupled in thalassemic Ca^{2+}/Mg^{2+} -ATPase was confirmed by examination of the Ca^{2+}-dependent property of the enzyme (Figure 39-44) and experiments on assay of the ATPase activity without addition of Ca^{2+} ions and calmodulin into the coupled enzyme medium (Figure 47). The results showed that most of the measurable ATPase activity in thalassemia samples were represented by the Ca^{2+} /calmodulin-independent ATPase. Cell fractionation experiments

(Figure 45-46) indicated that the Ca^{2+} /calmodulin-independent ATPase activity was prominent in the light ($\rho < 1.090$) cell fraction which usually is constituted of young and abnormal red cells.

The malfunction of the thalassemic erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase was also supported by studies on the formation of phosphoenzyme intermediate. As stated above, phosphorylation reaction is essential for the coupling of the ATPase activity to the Ca^{2+} translocation. Loss of the ability to form phosphoenzyme in all types of thalassemic erythrocyte Ca^{2+} /Mg^{2+} -ATPase (Figure 58-60) indicated that the Ca^{2+} pump was defective in these erythrocytes.

The inhibitory effects on the erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase by heme iron (124), non heme iron (125), and conditions that generate oxygen free-radicals (126-131) have been reported. The effects of oxygen free- radicals generating conditions on decreased response of the erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase to calmodulin have also been observed by several investigators (132-134). Those reports are consistent with the observations in this study.

This possible mechanism for the cause of damages in thalassemic erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase was confirmed by studies on the effects of the oxidative stress upon normal red cells. Thus, intact normal erythrocytes were exposed to various oxidants and the enzyme activity was examined following membrane preparations. The Arrhenius profiles of the enzyme from diamide and p-chloromercuribenzoate-treated erythrocytes

indicated that neither the formation of covalent crosslinkage nor the reduction of sulfhydryl content of the enzyme exerted changes to the Arrhenius properties similar to that of the thalassemic enzyme (Figure 30, 31). These observations were consistent to those reported by Nirdnoy et al (181) that the changes in the Arrhenius property were not associated with enzyme cross-linking. On the other hand, the effects of phenazine methosulfate, tert-butylhydroperoxide, phenylhydrazine, and xanthine oxidase generated Arrhenius profiles (Figure 33,35,37) and Ca^{2+} dependence response (Figure 48-55) which resembled those observed in the thalassemia samples, indicating that alterations to the properties of Ca^{2+} /Mg^{2+} -ATPase in thalassemic erythrocytes were the consequences of oxygen free-radicals.

In an aqueous solution, the oxidising agents xanthine oxidase and tert-butylhydroperoxide generate peroxyl radicals, $\cdot HO_2^-$ (126), while phenazine methosulfate and phenylhydrazine generate superoxide radicals, $\cdot O_2^-$,(126, 162). All cause lipid peroxidation of the phospholipids in the bilayer. Evidences for the existence of membrane lipid peroxidation have been reported in thalassemic erythrocytes (60, 76) and other hereditary hemolytic anemia (119, 123, 163). The effects of lipid peroxidation in normal and sickle erythroytes exposed to tert-butylhydroperoxide on the inhibition of Ca^{2+} pump ATPase have also been reported (127, 129).

The mechanism of oxidative damage has been proposed to take place via the generation of reactive oxygen -free radicals species such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl free-radical ($\cdot OH$), following attachment of unstable hemoglobin on the erythrocyte membrane. The reaction can be catalysed by either Fe or Cu ions and is generally known as the "Fenton reaction" (58). These oxygen free-radicals can then initiate the chain reactions of membrane lipid peroxidation (59) which may directly cause alterations in the topography and the function of the Ca^{2+}/Mg^{2+} -ATPase.

Evidences from site-directed mutagenesis and covalent modification studies on erythrocyte membrane Ca^{2+} /Mg^{2+} -ATPase showed that modification of one of the specific 4 glutamyl residues locating in the transmembrane helices 4, 5, 6 and 8, and most modifications near the phosphorylation site result in loss of both Ca^{2+} transport and Ca^{2+} -induced phosphorylation (164). So, the observations in this study that the thalassemic erythrocyte Ca^{2+} pump lost the ability to form phosphoenzyme (Figure 58-60) suggested that covalent modification of specific essential amino acid residue(s) may have caused an inactivation of the enzyme.

Another possible mechanism of inactivation of the thalassemic erythrocyte membrane Ca^{2+}/Mg^{2+} -ATPase is the oxidative cleavage of the polypeptide. The effects of oxygen free-radicals on the oxidative degradation of protein have been reported by several investigators. As studied by Davies and colleagues (165, 166, 167), the

exposure of proteins to oxygen free-radicals induced charge changes, tryptophan loss, and bityrosine production. The hydroxyl radical-modified proteins are rapidly recognized and degraded by erythrocyte-originated, soluble, Ca^{2+}/ATP-independent (nonlysosomal) proteolytic enzyme. Non-enzymatic cleavage of protein by oxygen free-radicals have also been reported for the enzymes lactate dehydrogenase (168) and superoxide dismutase (169). Evidences for the degradation of membrane skeletal proteins had also been observed in this study (Figures 58a, 59a, and 60a). Thus, the possible role of oxygen free-radical-induced proteolytic cleavage of the Ca^{2+} /Mg^{2+} -ATPase in thalassemic erythrocytes and oxygen free-radical-exposed erythrocytes resulting in the removal of the calmodulin-binding domain and the decoupling of the ATPase and the Ca^{2+} translocation activities is also postulated in this study.

In this study, attempt to purify the Ca^{2+}/Mg^{2+}-ATPase from the phenazine methosulfate-treated erythrocytes using calmodulin affinity chromatography was also performed. The experiments were not successful for no enzyme could be bound to the column (data not shown). These experiments clearly indicate that oxygen free-radicals induce loss of the calmodulin-binding domain of the Ca^{2+} pump-ATPase.

Besides inactivation of the Ca^{2+} pump ATPase, the effects of oxygen free-radicals on changes in the Ca^{2+} permeability of the erythrocyte membrane has also studied. The mechanism of Ca^{2+} influx in erythrocytes

has not been well characterized. Varecka and Carafoli (93) proposed that the influx of Ca^{2+} into the erythrocytes is a carrier-mediated process. The channel is verapamil-sensitive and is possibly driven by the discharge of the transmembrane K^+ gradient. Study in sickle cell disease (158) revealed that oxidative condition induced by deoxygenation stimulates an increase in Ca^{2+} permeability. The process could result from both the activation of a Ca^{2+} channel and of a transport system for cations involving interactions between polymerized hemoglobin S, band 3 and other membrane components. Endocytosis appears to play only a minor role in the Ca^{2+} uptake of deoxygenated sickle cells. The effects of lipid peroxidation on perturbations of the functions of verapamil- and nifedipine-sensitive Ca^{2+} channels resulting in a net influx of Ca^{2+} ions have been studied in isolated rat hepatocytes (170). The Figures 61 and 62 in this study showed that phenazine methosulfate induced perturbations of the erythrocyte membrane permeability. The Ca^{2+} influx was increased, but simple diffusion was decreased.

Taking these data together, the results suggested that oxygen free-radicals could induce both inactivation of the Ca^{2+} pump ATPase and increase in Ca^{2+} permeability in the erythrocyte membrane. This may explain the intracellular Ca^{2+} elevation in the thalassemic erythrocytes. This mechanism is consistent with a recent report studied in deoxygenated sickle erythrocytes (157).

CHAPTER 5

SUMMARY

The origin of the pathology in thalassemia disease is based on the defects of the globin genes. The ultimate consequence in the thalassemic erythrocytes is an overdestruction of the red blood cells by the reticuloendothelial systems , leading to a shortened life-span of the cells (47). Recent literatures (31) reveal that the presence of defective globin genes in thalassemia results in an imbalance synthesis of hemoglobin. The excess unmatched globin chains are vulnerable to oxidation and prefer to attach to the membranes; the affinity for the α-globin is more prominent than that of the β-globin. The precipitation of methemoglobin results in the release of oxidised heme (hemin) to the membrane which, in turn, leads to the generation of reactive oxygen free-radicals (59). The phenomena result in the thalassemic erythrocytes to be under condition of oxidative stress.

Many diseases of hereditary hemolytic anemia (76, 119, 123, 162) and also in senescent erythrocytes (172, 173), the red blood cells are under oxidative stress and contain increased level of Ca^{2+} ions. Studies on intact normal human erythrocytes (85-91) have shown that experimental overloading of Ca^{2+} ions have deleterious effects on the topography of erythrocyte membranes. The cells show changes in distributions of phospholipids components of the lipid bilayer and degradation of the membrane cytoskeletons. Membrane vesiculations have also been observed. These

alterations are also the characteristics of the thalassemic erythrocyte membranes.

The purpose of this study was focused on the mechanism(s) of accumulation of Ca^{2+} ions in the red blood cells. Regulation of Ca^{2+} ions concentration in erythrocytes is accomplished byvery low influx of the ions and effective extrusion of the ions by the membrane-bound Ca^{2+} /Mg^{2+}-ATPase (84). The effects of oxygen free-radicals on perturbations of the two intracellular Ca^{2+}-regulating mechanisms had been investigated in this study. The results can be summarized as followings:

1. The changes in Arrhenius properties and the alteration in Ca^{2+} pump-ATPase from being Ca^{2+}/calmodulin-stimulated to Ca^{2+}/calmodulin-independent, suggested that an uncoupling had occured in the thalassemic erythrocyte membrane Ca^{2+}/Mg^{2+} -ATPase.

2. The defective function of the thalassemic erythrocyte membrane Ca^{2+}/Mg^{2+}-ATPase was confirmed by the increase in Ca^{2+}/calmodulin-independent ATPase activity but a decrease in the ability to form the active phosphoenzyme intermediate.

3. The effects of oxidising agents have been investigated in both intact normal human erythrocytes and normal membrane preparations. Diamide (a cross-linking agent), p-chloromercuribenzoate (a sulfhydryl agent), phenazine methosulfate and phenylhydrazine (which produce superoxide), and tert-

butylhydroperoxide and xanthine oxidase (which produce peroxide) have been used in this study.

Diamide and p-chloromercuribenzoate inhibited both calmodulin-unstimulated and calmodulin-stimulated ATPase activity of the Ca^{2+} pump-ATPase. The oxidants had no effects on the calmodulin response of the enzyme nor on the increase in Ca^{2+} /calmodulin-independent ATPase activity. The effects of oxygen free-radical generating oxidants on the Arrhenius properties and the Ca^{2+}/calmodulin-dependency of the Ca^{2+}/Mg^{2+}-ATPase resembled those observed in the thalassemic erythrocyte enzyme.

4. Perturbations of the erythrocyte membrane permeability showed reduction in simple diffusion but increase in Ca^{2+} permeability.

The conclusion that can be made was that the presence of oxygen free-radicals in thalassemic erythrocytes have effects on both the decrease in the Ca^{2+} efflux and the increase in Ca^{2+} influx mechanisms. These perturbations are responsible for the accumulation of Ca^{2+} ions in these pathologic erythrocytes. Figure 63 is a diagram summarizing the findings.

PROPOSED MECHANISMS OF CALCIUM ACCUMULATION IN THALASSEMIC RED BLOOD CELLS BY OXYGEN FREE-RADICALS

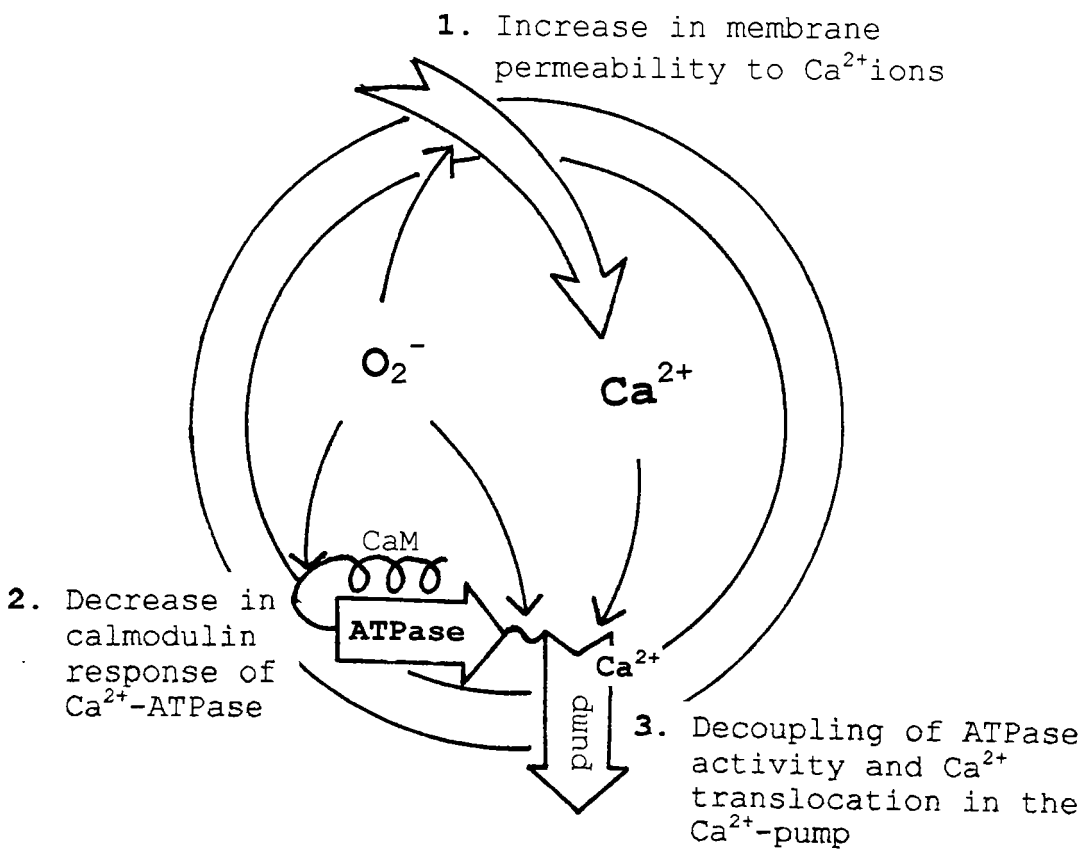


Figure 63: Summary of the effects of oxygen free-radicals on perturbations of the calcium homeostasis in human erythrocyte.

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APPENDIX

List of patients under investigation

	Name	Diagnosis	File No.
1.	P. Muangnoi.1.	Homozygous β-thal	8225-27
2.	P. Muangnoi.2.	Homozygous β-thal	8225-27
3.	V. Sornsoonthorn	HbH	25-1547
4.	K. Pichaikarn	β-thal/HbE	28-621
5.	V. Deejongcharoen	HbH/HbCS	102/32-2533
6.	S. Sawaengchai	Homozygous HbCS	28-276
7.	U. Patanapukdee	β^{4bp}-thal/HbE	14-1354
8.	K. Pimpa	HbH/HbCS	30-930
9.	T. Insrimuang	HbH	35-3314
10.	S. Sawangruang	β-thal/HbE	34-631
11.	S. Angthong	HbH	20-1377
12.	C. Tang	β-thal/HbE; spx	45-2509
13.	N. Jaibarn	β-thal/HbE	31/2515
14.	J. Junklom	β-thal/HbE; spx	11/2532
15.	P. Tongterm	β-thal/HbE; spx	75/26-1394
16.	N. Seangchin	β-thal/HbE; spx	60/2525-1349
17.	W. Seangchin	β-thal/HbE; spx	60/2525-1349
18.	O. Uthaipat	β-thal/HbE	21/2526
19.	S. Uthaipat	β-thal/HbE; spx	21/2526
20.	J. Klomlung	β-thal/HbE	65/35-2784
21.	S. Kraiprasit	β-thal/HbE; spx	-
22.	O. Maimann	β-thal/HbE; spx	104/2526
23.	B. Prakorbporn	HbH/HbCS	-
24.	C. Thumkoontod	HbH/HbCS	-
25.	T. Sritong	HbH	-

List of Patients (cont'd)

	Name	Diagnosis	File no.
26.	S. Chatcharoen	HbH/HbCS	-
27.	S. Sudta	HbH	-
28.	P. Kanchang	β-thal/HbE	-
29.	S. Senkaesin	β-thal/HbE	-
30.	S. Chumsri	β-thal/HbE	-
31.	S. Charoenpala	β-thal/HbE; spx	-
32.	S. Chomsak	β-thal/HbE; spx	-
33.	U. Wongswan	β-thal/HbE; spx	-