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# Porphyrin Compounds Inhibit $Ca^{2+}$-ATPase of Erythrocyte Plasma Membrane

**Abdul Hai[1], Jamal Alruwaili[2], Nadeem Kizilbash[3],**

Department of Biochemistry
Faculty of Medicine & Applied Medical Sciences
Northern Border University
Arar-91431, Saudi Arabia
*[1]synavia@address.com, [2]malkh1195@hotmail.com, [3] fsd707@gmail.com,*

**Syeda Huma H. Zaidi[4]**

Department of Chemistry
Faculty of Science
Northern Border University
Arar-91431, Saudi Arabia
*[4]humazaidi@gmail.com*

**Abstract:** *The regulation of $Ca^{2+}$ in human body is performed by $Ca^{2+}$-ATPase. The affinity of erythrocyte plasma membrane $Ca^{2+}$-ATPase* (*PMCA*) *was examined for three different porphyrin compounds, Tetraphenylporphinesulfonate* (*TPPS*), *5,10,15,20-Tetrakis* (*4-sulfonatophenyl*) *porphyrinato Iron*(*III*) *Chloride* (*FeTPPS*) *and 5,10,15,20-Tetrakis* (*4-sulfonatophenyl*) *porphyrinato Iron*(*III*) *nitrosyl Chloride* (*FeNOTPPS*), *by enzymatic assays. The plasma membrane $Ca^{2+}$-ATPase was inhibited by all three of these compounds resulting in an increase in intracellular calcium.*

**Keywords:** *Plasma membrane $Ca^{2+}$-ATPase, $Ca^{2+}$-ATPase inhibitors, Porphyrin derivatives, enzymatic assays, erythrocyte membrane.*

## 1. INTRODUCTION

$Ca^{2+}$ is an important signaling molecule, regulating cell cycle, metabolism, structural integrity, motility and volume **[1,2]**. Most of the $Ca^{2+}$ in cytosol is bound by $Ca^{2+}$-binding proteins, phospholipids and inorganic phosphate. Bound total intracellular $Ca^{2+}$ in erythrocytes (RBCs) reaches 5.7 μM **[3]**. Free $Ca^{2+}$ concentration under physiological conditions is 30 to 60 nM **[4]**. A gradient of 40,000-fold is maintained by the plasma membrane $Ca^{2+}$-ATPase (PMCA) between the cytosol and blood plasma **[4]**. This gradient may be used for signaling purposes **[5]**. An increase in internal $Ca^{2+}$ leads to changes in cell shape and volume, cellular rigidity and hemolysis **[6]**. Such changes arise from interactions of $Ca^{2+}$ with various molecular targets.

$Ca^{2+}$-ATPases are a group of enzymes responsible for active transport of cations across the cell membrane. The plasma membrane $Ca^{2+}$-ATPase (PMCA) is a P-type ATPase that participates in $Ca^{2+}$ signaling in erythrocytes **[7]**. Its abundance is ~0.1% of the total protein content in erythrocyte plasma membrane. In humans, the presence of the B-splice isoform of PMCA has been demonstrated earlier **[8]**. This protein is composed of 1220 amino acids forming ten transmembrane domains, two intracellular loops containing ATP-binding and phosphorylation sites and inward-facing *N*- and *C*-termini. The *C*-terminus contains a $Ca^{2+}$ Calmodulin-binding domain, phosphorylation sites and a PDZ-binding domain **[9]**.

Biological aging is a complex process, featuring mechanisms of age-related physiological and molecular changes **[10]**. Age-dependent changes of motor function, such as slowing of movement and muscle weakness, are associated with increased muscle contraction **[11]**. The accumulation of covalently modified proteins is an important feature of biological aging. The activity of

erythrocyte $Ca^{2+}$-ATPase typically decreases during aging **[12]**. The present work was undertaken to evaluate concentration-dependent effect of the three Porphyrin compounds, Tetraphenylporphinesulfonate (TPPS), 5,10,15,20-Tetrakis (4-sulfonatophenyl) porphyrinato Iron(III) Chloride (FeTPPS) and 5,10,15,20-Tetrakis (4-sulfonatophenyl) porphyrinato Iron(III) nitrosyl Chloride (FeNOTPPS), on plasma membrane $Ca^{2+}$-ATPase of erythrocytes by the use of enzymatic assays.

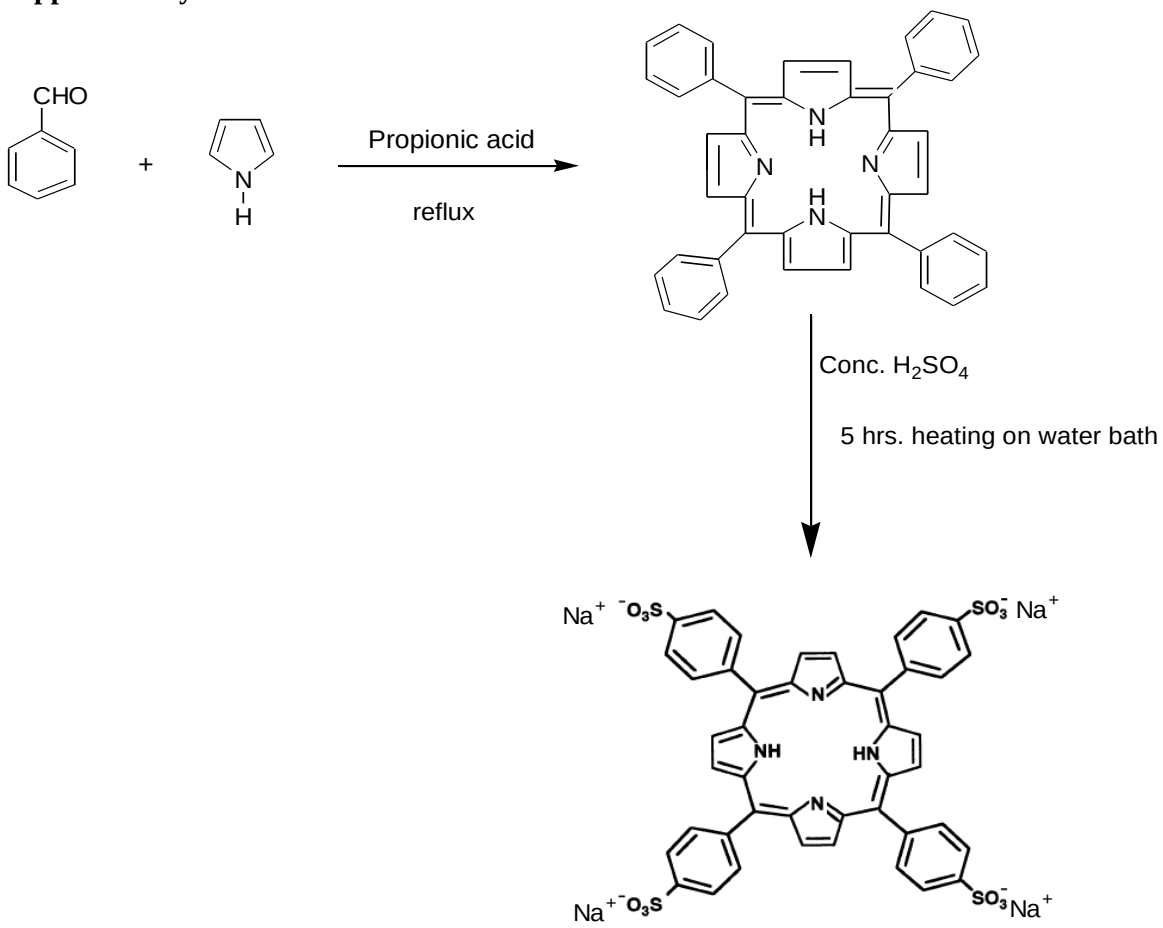
## 2. MATERIALS & METHODS

### 2.1. Synthesis of Porphyrin Compounds

One equivalent each of pyrrole and benzaldehyde were mixed together and then 1mL boron trifloroetherate was added rapidly. A calculated amount of p-chloranil was then added as an oxidant and then the solution was refluxed for one hour. The solvent was evaporated and the product was separated using column chromatography using initially 1: 2 ratio of DCM and petroleum ether and gradually changing the ratio to 1: 1 (Scheme 1).

TPPS was synthesized according to the published literature procedure (Scheme 1) **[13]**. Pure TPP and concentrated $H_2SO_4$ were ground into a homogenous paste and 50 ml of concentrated $H_2SO_4$ was added. The mixture was heated in a steam bath for 4 hours and allowed to stand at room temperature for 48 hours. The filtrate was diluted with two volumes of distilled water and salt was precipitated with addition of acetone.

**Supplementary Material**

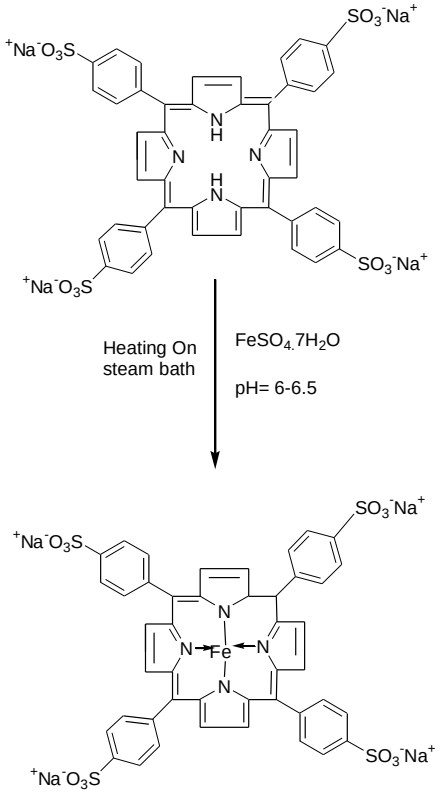



**Scheme1**. *Synthesis of TPP and TPPS.*

*Chemical Characterization: $C_{44}H_{30}N_4$; C, 85.90%; H, 4.92%; N, 9.12%; $^1$H NMR (400 MHz) ($CDCl_3$) $\delta$ 2.5-2.62 (br s, 2H, NH), 8.1-8.2 (dd, 8H, pyrrole), 7.6-7.7 (s, 20H, phenyl); UV-Vis (max.) 417, 513, 546, 589, 642 nm.*

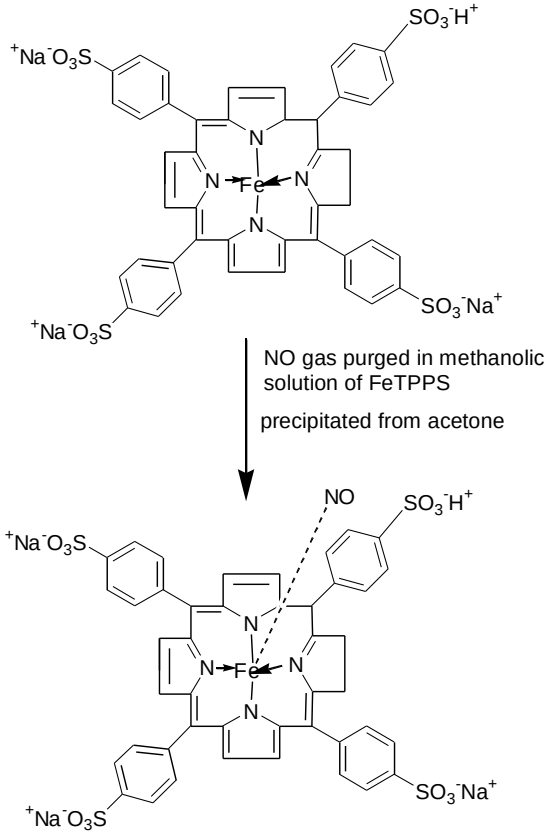
FeTPPS was also prepared by using published methods **[13]**. But a modification was made during the purification of the final product. Instead of following the purification process by cation-

exchange method, the classical method of the removal of iron using phosphate salt was adopted. Excess sodium sulfate and chloride present as impurities could be removed by dissolving the crude product in methanol and precipitating FeTPPS from solution by acetone (Scheme 2).




**Scheme2.** *Synthesis of FeTPPS.*

FeNOTPPS was prepared by using the laboratory synthesized FeTPPS (Scheme 3). Nitric oxide was bubbled in a solution of FeTPPS. The completion of the reaction was confirmed by the shift in Sǒ ret band in the UV-Vis spectrum from 394 to 420 nm.




**Scheme3.** *Synthesis of FeNOTPPS.*

*2.2 Collection of blood samples*:

The criteria for selection of subjects were the same as described earlier **[14]**. The study used blood samples from normal healthy subjects of both sexes that were divided into young (18-35 years; 32 subjects), middle (36-60 years; 31 subjects) and old (> 60 years; 26 subjects) groups.

### 2.2. Preparation of Erythrocyte Membranes

Venous blood (10 ml) was obtained from the research subjects by venipuncture and stored in heparin vials. The blood was centrifuged at 1800 × g for 10 min at 4°C. After collection of plasma, the erythrocytes were washed twice with cold phosphate buffer saline (0.9% NaCl, 10 mM $Na_2HPO_4$, pH 7.4). The erythrocyte membrane from leukocyte free red cells was prepared following the method of Marchesi and Palade **[15]**.

### 2.3. Determination of $Ca^2$+-ATPase Activity

The activity of $Ca^{2+}$-ATPase was determined as described in literature **[16]**. The reaction mixture was 2.25 ml in volume and it contained 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 18 mM Tris-HCl (pH 7.4), 0.1 mM ouabain, 0.1 mM EGTA, 0.2 ml of the membrane-containing 0.4 to 1.5 mg protein/ml and 0.2 mM $CaCl_2$. The reaction was initiated by the addition of 0.1 ml of 30 mM ATP at 37°C, and after 30 minutes, the reaction was stopped by adding 3.5 ml of a solution containing 0.5 M $H_2SO_4$, 0.5% ammonium molybdate and 2% SDS. The amount of released inorganic phosphate was estimated **[17]**. The measured $Ca^{2+}$-ATPase activity is expressed in terms of released μmole of inorganic phosphate (Pi) / hr/mg membrane protein at 37°C.

## 3. RESULTS & DISCUSSION

Human erythrocytes are typically used as a model system because they allow extrapolation of the acquired data to tissue cells. Investigation of inhibition of plasma membrane $Ca^{2+}$ ATPase (PMCA) by Porphyrin compounds provides useful information to understand the accumulation of calcium in the cytosol, a phenomenon that leads to cell death. Inhibition of the $Ca^{2+}$ ATPase has previously been shown to occur as a result of neutrophil-derived superoxide dismutation to $H_2O_2$ and diffusion of $H_2O_2$ into erythrocytes resulting in a Fenton-type reaction between oxyhemoglobin and $H_2O_2$ that produces hydroxyl radicals and ferryl radicals that cause fragmentation of the $Ca^{2+}$ ATPase **[18]**.

According to the kinetic model for the PMCA, the enzyme exists in two conformations, $E_1$ and $E_2$. $E_1$ has a high affinity for $Ca^{2+}$ and can be phosphorylated by ATP, whereas $E_2$ has a low affinity for $Ca^{2+}$ and can only be phosphorylated by inorganic phosphate ($P_i$). After binding of intracellular $Ca^{2+}$, $E_1$ can be phosphorylated by ATP to form the $E_1P$ intermediate. A change in conformation to $E_2P$ allows $Ca^{2+}$ to be released to the extracellular fluid from low affinity sites, followed by the hydrolysis of the phosphoenzyme to $E_2$ and the return to $E_1$ state **[19]**. During some stage of the reaction cycle, $Ca^{2+}$ becomes trapped in the enzyme when it is being transported from one side of the plasma membrane to the other **[20]**.

All the three compounds, TPPS, FeTPPS and FeNOTPPS were successfully synthesized (Schemes 1, 2 and 3). FeTPPS was prepared by using a modified version of the published method **[13]**. Instead of using the literature-described purification process of cation-exchange chromatography that can lead to demetallation and loss of yield, the classical method of the removal of iron using phosphate salt was adopted. Using this method, the yield of the pure product was increased to 90% (Scheme 2).

Enzymatic assays were performed to investigate the binding of PMCA and Porphyrin compounds (Table 1). To achieve this end, three Porphyrin compounds, Tetraphenylporphinesulfonate (TPPS), 5, 10, 15, 20-Tetrakis (4-sulfonatophenyl) porphyrinato Iron (III) Chloride (FeTPPS) and 5, 10, 15, 20-Tetrakis (4-sulfonatophenyl) porphyrinato Iron (III) nitrosyl Chloride (FeNOTPPS) were tested against PMCA. The results showed that TPPS forms the most stable complex with $Ca^{2+}$-ATPase (Table 1). It inactivates the PMCA completely, which results in a significant rise in $Ca^{2+}$ inside the cells.

**Table1.** *$Ca^{2+}$-ATPase activity after in vitro treatment with TPPS, FeTPPS and FeNOTPPS*

| **$Ca^{2+}$-ATPase Activity*** | | |
|---|---|---|
| **Control** | | 0.086 ± 0.003 |
| **Compound** | **Concentration(M)** | |
| TPPS | $10^{-10}$ | 0.112 ± 0.037 |
| | $10^{-9}$ | 0.135 ± 0.023 |
| | $10^{-8}$ | 0.173 ± 0.031 |
| | $10^{-7}$ | 0.208 ± 0.021 |
| | $10^{-6}$ | 0.233 ± 0.026 |
| | $10^{-5}$ | 0.250 ± 0.031 |
| | $10^{-4}$ | 0.275 ± 0.011 |
| | $10^{-3}$ | 0.322 ± 0.035 |
| FeTPPS | $10^{-10}$ | 0.093 ± 0.008 |
| | $10^{-9}$ | 0.112 ± 0.020 |
| | $10^{-8}$ | 0.137 ± 0.012 |
| | $10^{-7}$ | 0.153 ± 0.032 |
| | $10^{-6}$ | 0.179 ± 0.025 |
| | $10^{-5}$ | 0.199 ± 0.039 |
| | $10^{-4}$ | 0.221 ± 0.020 |
| | $10^{-3}$ | 0.260 ± 0.017 |
| FeNOTPPS | $10^{-10}$ | 0.083 ± 0.015 |
| | $10^{-9}$ | 0.100 ± 0.012 |
| | $10^{-8}$ | 0.118 ± 0.017 |
| | $10^{-7}$ | 0.136 ± 0.020 |
| | $10^{-6}$ | 0.164 ± 0.016 |
| | $10^{-5}$ | 0.184 ± 0.033 |
| | $10^{-4}$ | 0.204 ± 0.021 |
| | $10^{-3}$ | 0.234 ± 0.026 |

**$Ca^{2}$+-ATPase activity is expressed in terms of μmole of inorganic phosphate (Pi) released/hr/mg membrane protein at 37oC.*

## 4. CONCLUSION

Enzymatic assays revealed that interactions of TPPS with $Ca^{2+}$-ATPase are energetically more favorable than similar interactions with FeTPPS and FeNOTPPS. It is possible that binding of TPPS to $Ca^{2+}$ -ATPase locks the enzyme in a confirmation that inhibits enzyme action. In addition, the Porphyrin derivatives may bind at a site of the cell membrane away from the active site and cause differential changes in membrane properties, which can, in turn, affect the enzymatic activity.

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## AUTHORS' BIOGRAPHIES

**Dr. Abdul Hai** received his Master's degree from University of Allahabad, India. His doctoral degree was awarded in Biochemistry in 2002 from IIT-Kanpur in India. He is presently working as an Assistant Professor of Biochemistry at Northern Border University (Arar, Saudi Arabia). He has previously worked as an Assistant Professor at Garyounis University (Benghazi, Libya). His work experience also includes Postdoctoral Fellowship at Max Planck (Germany) and University of North Carolina (Raleigh, U.S.A.). He has published fifteen papers in international journals.

**Dr. Nadeem Kizilbash** received his Bachelor's degree in Chemistry from Longwood College (U.S.A.). His Master's degree was also in Chemistry from Washington University (St. Louis, U.S.A.). His doctoral degree was awarded in Biophysics from Boston University (U.S.A.). He is presently working as an Assistant Professor of Biochemistry at Northern Border University in Arar, Saudi Arabia. He has previously worked as an Assistant Professor at Quaid-i-Azam University in Pakistan. He has published fifteen papers in various journals on topics as diverse as Proteomics, Protein Structure, Drug Delivery and Gene Polymorphism.

**Dr. Jamal Alruwaili** received his doctoral degree in Biochemistry in 2011 from the University of Portsmouth in U.K. The topic of his dissertation was: "Serum Proteomic Analysis of Prostate Cancer Progression". He is presently working as the Vice Dean of the Faculty of Applied Medical Sciences at Northern Border University in Arar, Saudi Arabia. He has previously worked as a Scientist at King Faisal Specialist Hospital in Riyadh, Saudi Arabia. He has published papers in international journals on topics as diverse as Proteomics, Protein Structure, Molecular Docking and Drug Delivery.