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Na^+/K^+ - ATPase ACTIVITY IN NORMAL AND SICKLE CELL ERYTHROCYTES

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Abstract

Na^+/K^+ - ATPase activity, was found to be highest in HbSS, followed by HbAS and then HbAA in all the samples analyzed. The enzyme activity was found to increase with increase in temperature and then optimum temperature was found to be 40^oC in all the genotypes. The enzyme was also found to obey hyperbolic kinetics. The Km of the enzyme was found to be 0.38mM, while the Vmax was 305.8 µmol Pi/Mg Pr/hr. The enzyme also had activation energy (Ea) of 0.14 Kcal/mol. The differences in the enzyme activity for the various genotypes tested would mean that the rate of Na^+, K^+ uptake by the active transport mechanism would be in the order, HbSS > HbAS > HbAA.
Keywords: Na^+/K^+ - ATPase, Enzyme, Genotypes, Activation Energy, Kinetics, Temperature, Vmax, Km.

1. Introduction

It is agreed that the shape of the erythrocytes, a biconcave disc is such as to provide an optimum surface to volume ratio and thus is best suited for its role in respiratory exchange. The cell is not rigid when it come in contact with chemicals or meets an obstruction, it elongates or bends and then returns to its original conformation.
Enzymatic activities in the erythrocyte membrane is necessary for the maintenance of normal membrane structure and functions, for the normal oxygenated haemoglobin and for the protection of haemoglobin against peroxidation. The maintenance of the erythrocyte's normal volume and shape is dependent on control of its internal ionic milleu.
The active cation transport pumps regulates the intracellular concentration of Ca^{2+}, Na^+-K^+ and Mg^{2+}. The energy required for the active transport of these cations are derived from the metabolism of glucose. This can be seen as the cyclic conversion of ATP to ADP and Pi and vice versa. The reaction process is catalyzed by a variety of enzymes called Adenosine triphosphatases (ATPases). The reaction catalyzed by these set of enzymes can be shown thus:
$ATP^{4+} + H_2O \rightleftharpoons ADP^{3+} + HPO_4^{2+} + H^+$
There are three different Adinosine triphosphatases in erythrocyte membranes: Ca^{2+}-ATPases, Na^+/K^+-ATPases and Mg^{2+}-ATPases, each activated or stimulated by one or more ions. Erythrocyte membrane ATPases are membrane bound enzymes that have transport function. They are vectorial enzymes in that their enzymes activities lead to the transport of ions in a certain direction.
Three classes of ATPases are known to date. These are found to fall into the "P", "V" and "F" classes. The "P" ATPase can be described as those which form covalently phosphorylated intermediate, (Schatzmann, 1982). The common examples of these class are Ca^{2+}-ATPase, Na^+/K^+-ATPase, Mg^{2+}-ATPase and H^+-ATPase.
Erythrocyte Na^+/K^+-ATPase like other membrane ATPase is a membrane bound enzyme that has a transport function which couples the free energy contained in ATP to translocate Na^+ and K^+ across the membrane (Skou, 1965). The enzyme belongs to "P" type classification (Perdersen & Carafoli 1987). In conjunction with erythrocyte ATPases it functions primarily in the active transport of mineral across the erythrocyte membrane and this is essential if the call membrane stability is to be maintained.
In animal cells the major ion-motive ATPase is the ovabain sensitive sodium pump (Na^+/K^+-ATPase) which was first described by(Skou, 1957) in crab nerve. Na^+-K^+ has also been purified from tissues like kidney medulla, intestinal membrane and brain with Molecular weight of 95,000.
In most animal cells, the intracellular K^+ concentration is relatively high and constant. It falls between 120 and 160 mm, whereas that of Na^+ is less than 10mm (White *et al*. 1978). In contrast the extracellular fluid contains a relatively high Na^+ concentration about 150mm while K^+ is about 4mm. A significant concentration gradient of these two ions exists across the membrane of cells.

The constancy of the high internal K^+ concentration is maintained by the energy requiring extrusion of Na^+ out of the cell and its replacement by K^+ promoted by an active transport system, the Na^+/K^+-ATPase. The relatively high concentration of internal K^+ are essential for several processes, example, in protein biosynthesis as demonstrated by (Nomural *et al*. 1974), the reassembly of 16S RNA and ribosomal proteins into 30S RNA could only occur in high concentration of K^+, etc. Na^+ also plays an indispensible role in the transport of glucose into the cell. In fact inward glucose transport is optimal when there is a large inward gradient of Na^+ into the cell.
The specific ratio of Na^+ to K^+ transportation based on reports from experiments on red blood cell membrane and squid axon is 3:2 (Lehninger, 1977).
The enzyme is inhibited by cardiac glycosides including ovabain, digitoxin, digitoxigenin, digitonin and antibiotics like oligomycin (Schild, 1979; Doherty *et al*. 1982).

2. Materials And Methods

Instruments Used
Corning Colorimeter 253
Bench centrifuge (MSE, MINOR 35)
pH Meter (Pw 9414, PYE UNICAM, PHILIPS ENGLAND)
Water bath (Grant Instruments Cambridge Ltd.) Drying Oven, Refrigerator, Weighing Balance.

2.1. Blood Samples – All blood samples, Normal blood (HbAA), Sickle cell trait blood (HbAS) and Sickle cell blood (HbSS) were collected from Arico Medical Diagnostic Laboratory, Emekuku Street, Port Harcourt, in reagent bottles containing anticoagulant (0.05ml EDTA).

2.2. Preparation of 10mM tris-HCl Buffer pH 7.4
0.01M tris base was prepared by dissolving 1 gram of tris (hydroxyl methyl) aminomethane (BDH Chemicals Ltd. England) in 250ml of distilled water. 0.01M HCl was also prepared by mixing 21.5ml of concentrated HCl (BDH Analar) in 250ml of distilled water. Some quantity of the tris base was placed inside a beaker and electrodes of the pH meter immersed into it. Some quantity of the 0.01M HCl was added until the pH meter read 7.4. The Tris HCl buffer prepared was put in a reagent bottle and stored in the fridge.

2.3. Preparation of 1M NaH_2PO_4
31.75g of NaH_2PO_4 Salt (BDH Chemicals Ltd. England), was weighed and dissolved in 250ml of distilled water and the solution prepared was stored in a reagent bottle in the locker.

2.4. Preparation of 1% $CuSO_4.5H_2O$
10g of $CuSO_4.5H_2O$ (BDH Chemicals Ltd. England) was weighed and dissolved in 1 litre of distilled water and stored in a bottle in the locker.
2.5. Preparation of Na^+-K^+ Tartarate
20g of Na^+ - K^+ tartarate (BDH Chemicals Ltd. England) was dissolved in distilled water in a beaker and the solution made up to 1 litre with the same distilled water.

2.6. Preparation of Na_2CO_3 in 1M NaOH
20g of Na_2CO_3 (BDH Chemicals Ltd. England) and 4.0g of NaOH pellets (BDH Chemicals Ltd. England) were dissolved in distilled water and the solution made up to 1 litre with the same distilled water.

2.7. Preparation of Alkaline Copper Solution
This was prepared fresh just before use by adding 100ml of 2% Na_2CO_3 in 0.1M NaOH solution to 1.0ml of 2% Na^+-K^+ Tartarate to 1.0ml of 1% $CuSO_4.H_2O$ stirring well. The sequence of addition of these reagents described above was strictly followed.

2.8. Preparation of 9% Ascorbic Acid
22.5g of ascorbic acid (BDH Chemicals Ltd. England) was dissolved in distilled water in a beaker and the solution made up to 250ml mark in a measuring cylinder and stored in a bottle in the fridge.

2.9. Preparation of Ammonium Molybdate Solution
2.0g of ammonium molybdate (May and Baker, Lab. Chemical, England) was weighed in a tube. It was then transferred into a measuring cylinder and 9.0ml of concentrated sulphuric acid (H_2SO_4) (BDH Chemicals Ltd. England) was added. The mixture was well shaken and made up to 310ml with distilled water and the solution stored in the fridge.

2.10. Preparation of 21.0mM $MgCl_2$
2.0g of $MgCl_2$ was weighed and dissolved in a small quantity of distilled water in a beaker and the solution poured into a measuring cylinder and made up to 1 litre mark with distilled water. The prepared solution was put in a bottle and stored in a locker.

2.11. Preparation of 17.5mM $CaCl_2$
1.9425g of $CaCl_2$ was weighed and dissolved in a small quantity of distilled water in a beaker and the solution made up to 1 litre with distilled water in a measuring cylinder. The mixture was stored in a bottle in the locker.

2.12. Preparation of Tris-EDTA-Borate Buffer pH8.6 (25^oC)
12.0g of tris (hydromethyl) aminomethane (Tris), 1.56g of disodium ethylene-diaminetetraacetate (EDTA), 0.92g of boric acid were dissolved in deionized water and diluted to 1000ml. the solution was stored at 5^oC.

2.13. Preparation of 5.0M NaCl pH 7.4
8.775g of NaCl salt was dissolved in a small quantity of distilled water and the solution made up to 1 litre with distilled water. Some quantity of the solution was placed inside a beaker and electrodes of the pH meter immersed into it. Some quantity of a dilute sulphuric acid (H_2SO_4) was then added until the pH meter read 7.4.

3. Standard Curve For Protein Determination (Lowry's Principle)

1g of an egg albumin powder was measured and a solution is made with distilled water and the solution made up to 1000mls with the same distilled water.
This was used as a stock standard. A dilution was made out of this by taking 1ml of the main stock and made up to 100ml of with distilled water. This was equivalent to 100mls of 1000mg/l egg albumin solution. The required volume (20,40,60,80 and 100µl) were taken out of this dilution solution prepared and made up to 100µl with distilled water in the test tubes.
5mls of the reagent mixture (Alkaline copper solution) was then added to each tube and allow them to stand for 10 minutes at room temperature. Then 0.5ml of the diluted folinciocalteau solution was added to the tubes and mixed immediately and allowed to stand for 30 minutes at room temperature. The optical density of the mixture in each tube was read at 700nm in a colorimeter using the blank to zero the instrument. The optical density (O.D) was then plotted against standard protein concentration as shown in the graph (Fig. 3.1).

3.1. Standard Curve For Phosphate Determination

The method of Fiske and Subburn (1925) was used. To a series of labeled test tubes in a rack were added varying volumes (0.02,0.04,0.06,0.08 and 0.1ml) of 1M NaH_2PO_4. The solution were then made up to a final volume of 1.0ml with the distilled water by adding correspondingly (0.98,0.96,0.94,0.92,0.90 and a blank of 1.0ml) of distilled water respectively. 1.0ml of ammonium molybdate solution was then added to each of the tubes and mixed. The solution mixture was allowed to stand for 10 minutes after which 1.0ml of ascorbic acid solution was added to the tubes and allowed to stand for 20 minutes for the colour to develop.
The optical density of the solution mixture in each tube was read using a colorimeter at 760nm wavelength. The optical density (OD) was then plotted against the standard phosphate concentration as shown in figure 3.2.

3.2. Preparation Of Erythrocyte Membrane Ghost

The erythrocyte ghost membranes were prepared from recently outdated human blood samples whose genotypes have been determined.

0.1ml of each of the blood samples were put into test tubes set up in a rack and 0.3ml of 0.15M NaCl pH 7.4 solution added to each tube. This is centrifuged at 10,000 g for about 5 minutes. The produced supernatant which was discarded and the procedure repeated for about 3 to 4 times.
5mM NaH_2PO_4 solution was added to the sediment and then centrifuged again for about 5 minutes. The supernatant was removed and the precipitate (ghost cell) washed once in 10mM Tris HCl (pH 7.7). This also produced supernatant which was discarded and the precipitate suspended in 3ml of distilled water and stored in the fridge for 12 hours before use.
Na^+, K^+ - ATPase Assay
Effect of substrate concentration: The assay medium contain 0.5ml of 21.0mM $MgCl_2$, 0.5ml of 17.5 mM $CaCl_2$, 0.5ml of tris-HCl buffer (pH 7.4), 0.5ml of distilled water and 0.5ml of ATP-Na_2.
The reaction was then started by adding 0.2ml of the ghost cell to the assay medium and the reaction mixture incubated for 1 hour at 37^{o}C in a water bath. The reaction was stopped after the incubation by adding 0.8ml of ice-cold 10% trichloroacetic acid (TCA). The mixture was allowed to stand at 40^{o}C for 20 minutes after which it was centrifuged at 4,000 g for 5 minutes. The supernatant produced was separated from the precipitate. The concentrations of inorganic phosphate released in the supernatant was determined using the Fiske and Subburn method (1925) as used in the calibration curve and the protein content in the precipitate was determined using the method of Lowry et al (1951) as used in the calibration curve.
This procedure was carried out at different ATP-Na_2 concentrations (hence at 1mM, 2mM, 3mM and 5mM concentrations) respectively.

3.3. Effect Of Temperature On Na^+ and K^+ - ATPase Activity

The assay procedure was also carried out at different temperatures. This was done by adding the assay solution (containing 0.5ml of 21.0mM $MgCl_2$, 0.5ml of 17.5 mM $CaCl_2$, 0.5ml of tris-HCl buffer (pH 7.4), 0.5ml of distilled water and 0.5ml of ATP-Na_2). Into six different test tubes set up in a rack and the ghost cells (0.2ml) added into each of them. All the six test tubes were incubated at 10^{o}C for 10 minutes after which the first test tube was removed and the reaction in it stopped by adding 0.8ml of 10% ice cold TCA. At 20^{o}C, the second test tube was removed, at 30^{o}C, 40^{o}C, 50^{o}C and 60^{o}C, the third, fourth, fifth and sixth test tubes were respectively removed and their reactions stopped by adding 10% ice cold TCA solution. Each tube was centrifuged at 10,000 g for about 5 minutes and the supernatant separated from the precipitate. The phosphate concentrations and the protein concentrations were determined in the supernatant and precipitate of each tube respectively.

4. Results

1. The results in table 3.1 compares the Na^+/K^+-ATPase activity in normal cell (AA), Sickle cell trait (AS) and Sickle cell (SS) blood samples. The HbAA has an activity of 246.30 ± 17.42, HbAS has an activity of 277.04 ± 29.14 while HbSS has an activity of 301.45 ± 35.16.
From the result obtained, Na^+/K^+-ATPase activity was observed to be highest in Sicklers, SS followed by AS and the least in AA samples.
2. The results obtained in table 3.2 shows substrate effect on Na^+/K^+-ATPase activity on normal and sickle cell erythrocyte. The enzyme activity increases as the substrate concentration is increased as shown on the graph (fig. 3.3). This plot was found to obey hyperbolic kinetics. The kinetic constants Km and Vmax were obtained using the Line Weaver Burk plot (fig. 3.4). The plot shows that the enzyme has an apparent Km of 0.38MM and a Vmax of 305.8 µmol Pi/mg Protein.
3. Table 3.3 shows the temperature dependence of Na^+/K^+-ATPase activity on normal and sickle cell erythrocyte. The result show an initial rise in enzyme activity as the temperature is increased up to a point where a further increase in temperature led to a rapid decrease in the enzyme activity against temperature indicating an optimal temperature of 40^{o}C. An Arrhenius plot of the temperature effect (fig. 3.6) gives as activation energy (Ea) of 0.10 cal/mol energy.

5. Discussion

From the result obtained, Na^+/K^+-ATPase activity was observed to be highest in sicklers SS, followed by AS and least in AA samples. It then follows that the rate of Na^+/K^+=ATPase activity in the active transport mechanism for various genotypes tested is in the order of HbSS > HbAS > HbAA.

The results are in line with the work done by Madan G. L. and David A. S. (1982), who reported that sickle cell red blood hemolysates had greater Ca^+-ATPase activity than normal hemolysates; they exhibited higher Mg^{2+} and Na^+/K^+-ATPase activities as well. It was also said in their work, "Thus, when studied under conditions that maximize enzyme activity, Ca^+-ATPase activity, like Mg^{2+} and Na^+/K^+-ATPase, is actually increased in SS red blood cell, probably due to the young red cell population present. The elevated cation levels in these cells are more likely due to an increased cation (Ca^{2+}, Mg^{2+}, Na^+, K^+) leak or abnormal cation binding, than to defective extrusion by the ATPase pump.

This result may not be the same with other project works carried out. However a possible suggestion as to the behaviour of the Na^+/K^+-ATPase activity in this work may probably be due to maintenance of blood viscosity by Na^+ and K^+. Viscosity in blood is particularly associated with sickle cell disease. The high Na^+/K^+-ATPase activity in SS can then be attributed to the enzyme trying to maintain the sickle shape of the cell. It can then be said that the low activity for Na^+/K^+-ATPase in AA were normal.

The results obtained in table 3.2 shows that the activity of Na^+/K^+-ATPase increases as substrate concentration is increased up to a level where further increase in substrate concentration shows a decrease in the enzyme activity (fig. 3.3). From this plot, the Line Weaver-Burk plot was made (fig. 3.4). The graph obeys hyperbolic kinetics.

Temperature dependence of Na^+/K^+-ATPase shows that the enzyme has an optimum temperature of 40^{o}C (fig. 3.5), at higher temperature there was observed drop in enzyme activity giving a graph of a cone shape.

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Result Tables And Figures

A. 1: Table Of Values For Protein Standard Curve

S/No.	Absorbance (OD)	Protein Conc. (mg/ml)
1.	0.000	0.00
2.	0.032	0.20
3.	0.045	0.40
4.	0.091	0.60
5.	0.120	0.80
6.	0.152	1.00

A. 2: Table of Values for Phosphate Standard Curve

S/No.	Absorbance (OD)	Phosphate Conc. (µmol)
1.	0.000	0
2.	0.064	20
3.	0.150	40
4.	0.204	60
5.	0.241	80
6.	0.312	100

A. 3: Table of Values for Na^+/K^+-ATPase Activity in HbAA

ATP (mM)	Phosphate Protein				Enzyme Activity (µmol Pi/mg, Pr/hr)
	O.D at 670 nm	Conc. (µmol)	O.D at 700nm	Conc. (mg/ml)	
0.00	0.000	0.00	0.000	0.00	0.00
1.00	0.030	112.00	0.018	0.50	224.00
2.00	0.057	121.00	0.034	0.51	237.30
3.00	0.081	147.00	0.043	0.58	253.40
5.00	0.100	165.00	0.039	0.61	270.50

A. 4: Table of Values for Na^+/K^+-ATPase Activity in HbAS

ATP (mM)	Phosphate Protein				Enzyme Activity (µmol Pi/mg, Pr/hr)
	O.D at 670 nm	Conc. (µmol)	O.D at 700nm	Conc. (mg/ml)	
0.00	0.000	0.00	0.000	0.00	0.00
1.00	0.034	130.00	0.020	0.54	240.70
2.00	0.067	146.00	0.036	0.55	265.50
3.00	0.093	163.00	0.044	0.58	281.00
5.00	0.115	199.00	0.037	0.62	320.90

A 5: Table of Values for Na^+/K^+-ATPase Activity in HbSS

ATP (mM)	Phosphate Protein				Enzyme Activity (µmol Pi/mg, Pr/hr)
	O.D at 670 nm	Conc. (µmol)	O.D at 700nm	Conc. (mg/ml)	
0.00	0.000	0.00	0.000	0.00	0.00
1.00	0.039	148.00	0.020	0.56	264.30
2.00	0.073	159.00	0.036	0.58	274.10
3.00	0.108	195.00	0.048	0.62	314.50
5.00	0.139	240.00	0.058	0.68	352.90

A 6:Results of Enzyme Dependency Temperature

Temperature (^oC)	Phosphate Protein				Enzyme Activity (µmol Pi/mg, Pr/hr)
	O.D at 670 nm	Conc. (µmol)	O.D at 700nm	Conc. (mg/ml)	
0	0.073	0.31	0.229	62.0	200.0
10	0.087	0.37	0.307	83.0	224.3
20	0.106	0.45	0.351	95.0	235.1
30	0.092	0.39	0.517	140.0	358.9
50	0.094	0.40	0.443	120.0	300.0
60	0.085	0.36	0.228	62.0	172.0

A. 7: Table of Values for Graph of V_O Against Substrate Concentration (Figure 3.3)

Substrate Conc. (mM)	V_O (µmol Pi/mg Pr/hr)
1	243.0
2	259.0
3	283.0
5	315.0

A 8: Table of Values for Line Weaver-Burk Plot (Figure 3.4)

$^1/_S$ $(mM)^{-1}$	$^1/V_O$ $(\mu mol\ Pi/mg\ Pr/hr)^{-1}$
1.0	4.1×10^{-3}
0.5	3.8×10^{-3}
0.3	3.5×10^{-3}
0.2	3.1×10^{-3}

The intersection of this graph at $^1/V_O$ axis gives

$^1/V_{max} = 2.85 \times 10^{-3}$ ∴ Vmax = 305.8 (µmol Pi/mg Pr/hr) Slope = $^{Km}/V_{max} = 1.25 \times 10^{-3}$ ∴ Km = 1.25×10^{-3} x 305.8 = 0.38 mM.

A. 9: Table of Values for Arrhenius Plot (Figure 3.6)

$^1/_T$ $((K^{-1})$	$^1/V_O$ $(\mu mol\ Pi/mg\ Pr/hr)^{-1}$
0.100	5.0×10^{-3}
0.050	4.5×10^{-3}
0.030	4.7×10^{-3}
0.025	2.8×10^{-3}
0.020	3.3×10^{-3}
0.016	5.8×10^{-3}

The Slope of the graph = $^{Ea}/_{2.303\ R}$ = 0.03 R is a constant = 1.987 cal/mol ∴ Ea = 0.03 x 2.303 x 1.987= 0.137 cal/mol

Figure 3.1: Standard Curve for Protein Determination (Lowry's Principle)

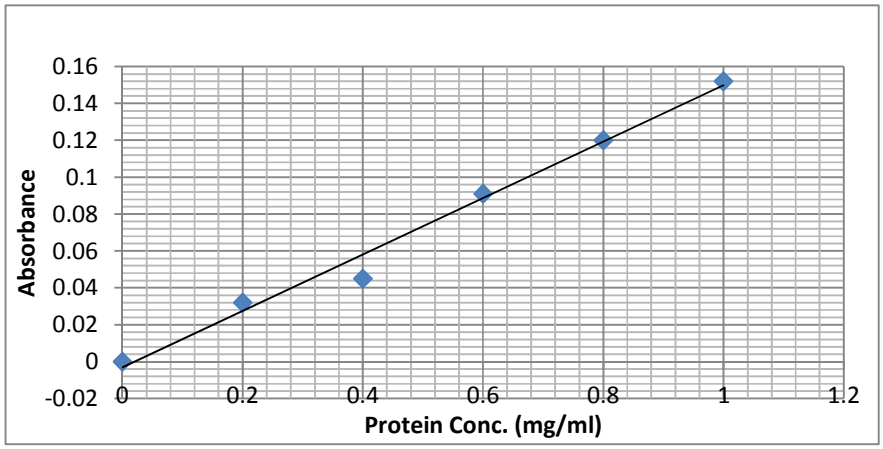


Figure 3.2: Standard Curve for Phosphate Determination (Fiske & Subburn Method)

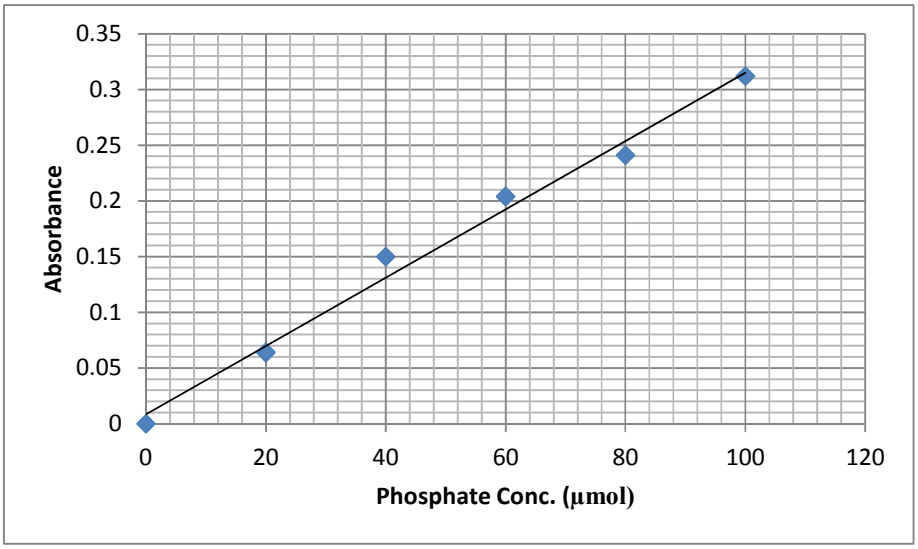
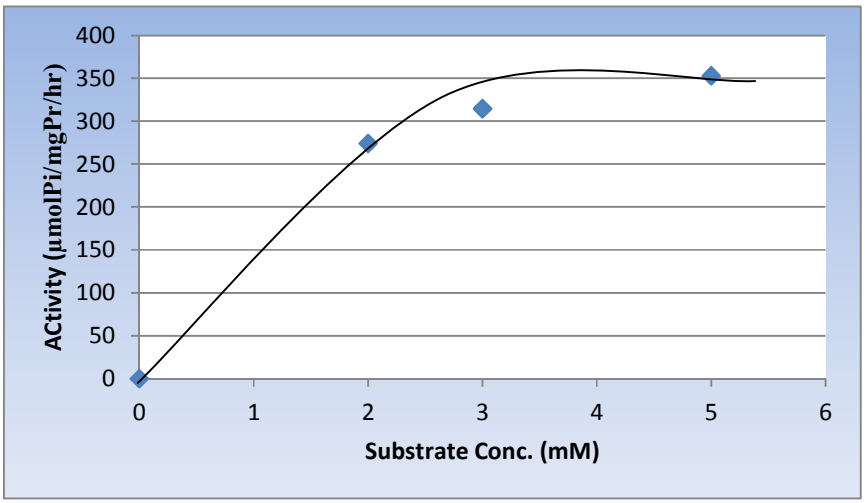


Figure 3.3: Effect of Substrate Conc. on Na^+/K^+-ATPase on Erythrocyte Membrane



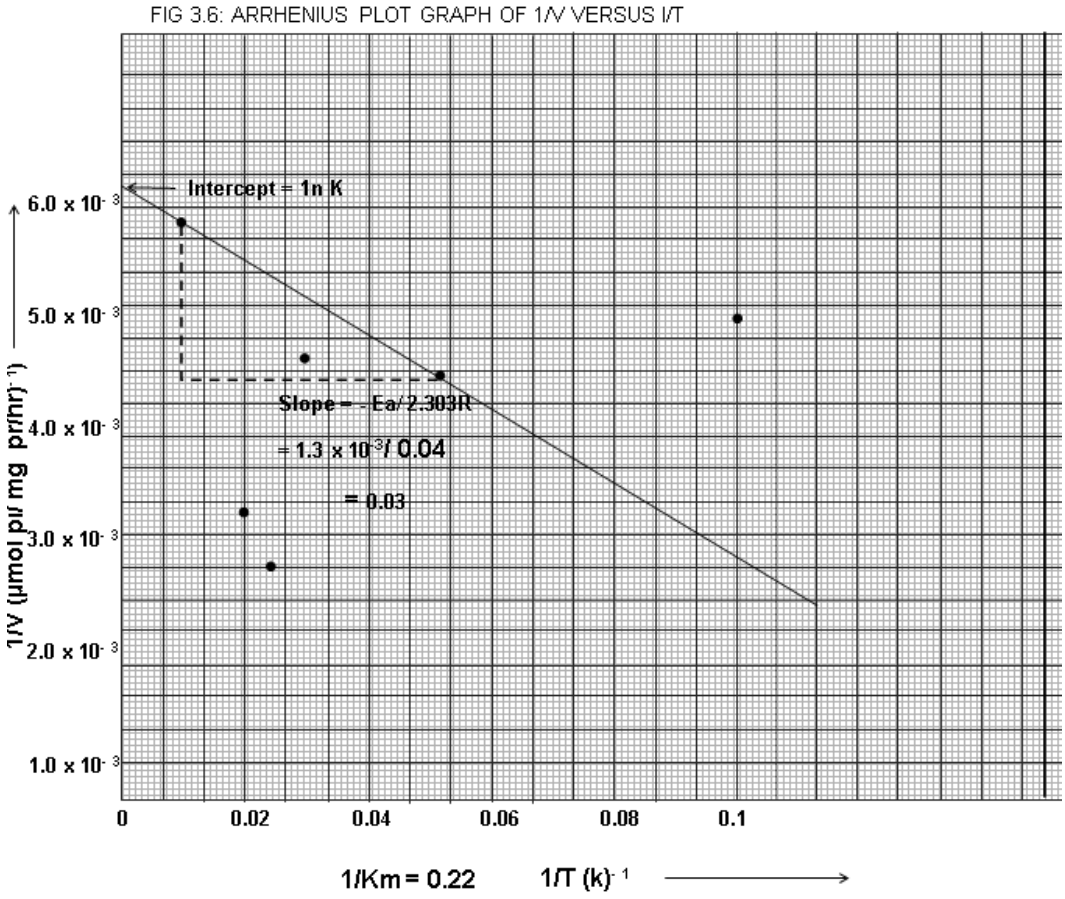


FIG 3.5: ARRHENIUS PLOT GRAPH OF 1/V VERSUS I/T

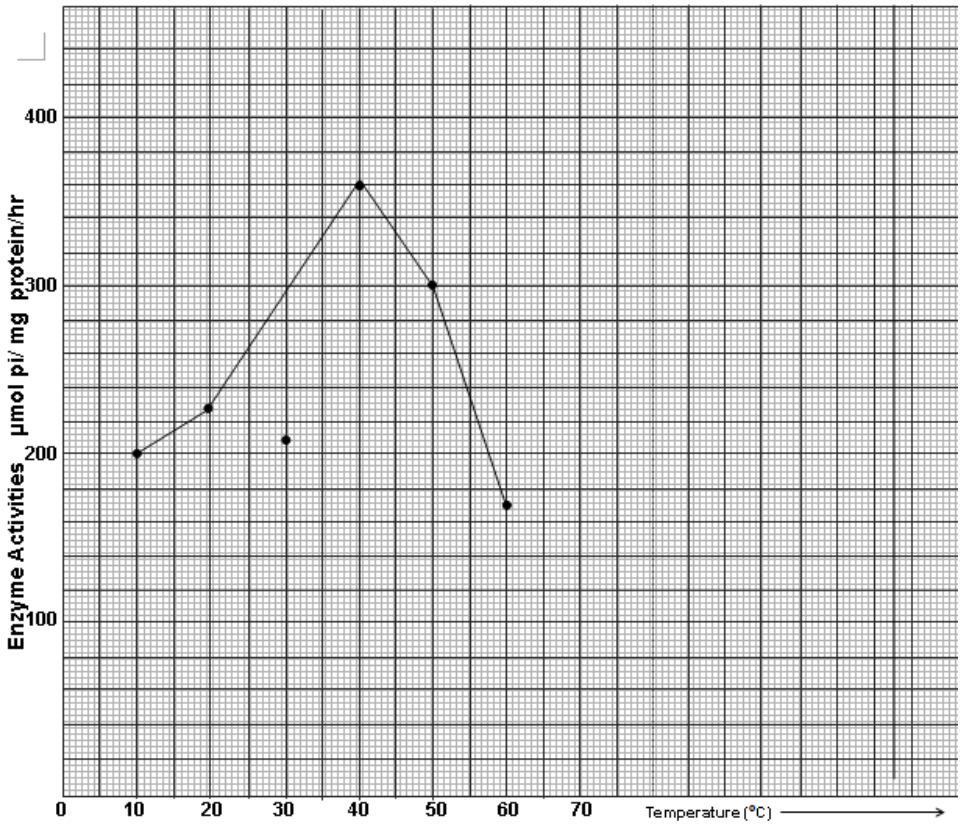
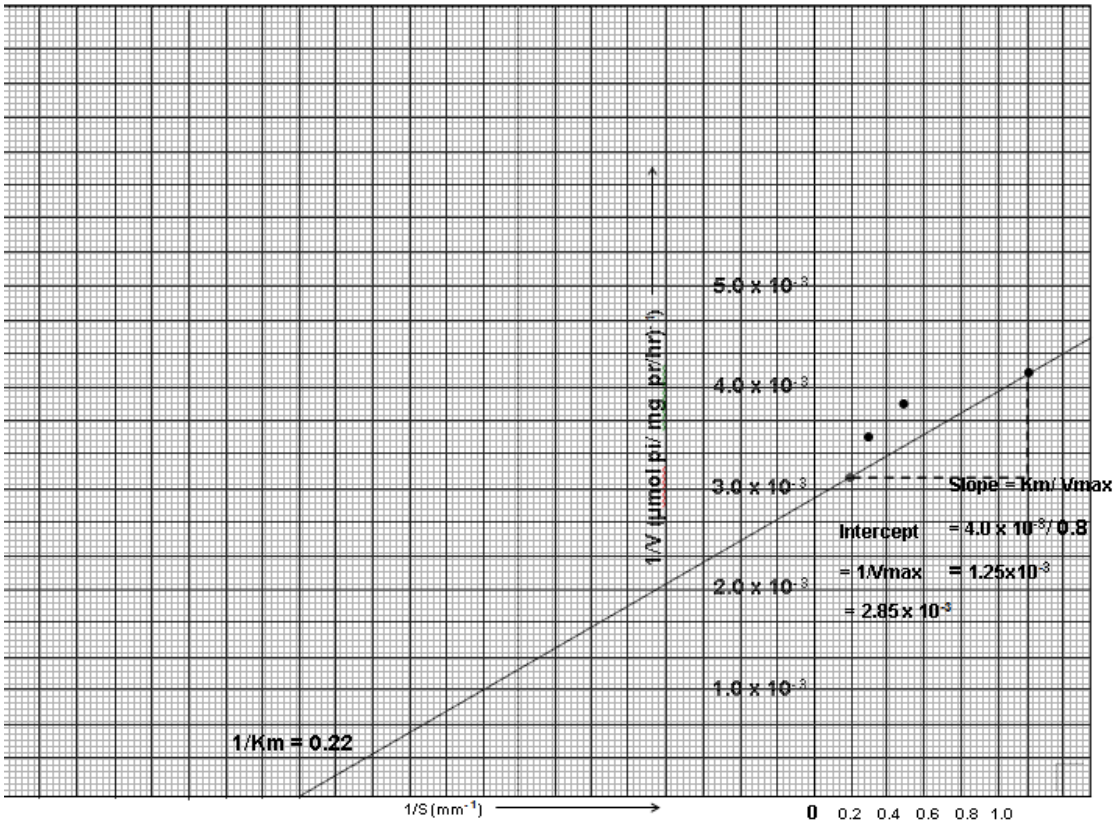


FIG 3.4: LINE WEAVER – BURK PLOT



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